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Ovarian cancer is the fourth leading cause of cancer-related death in U.S. women. This program project approaches the ovarian cancer problem by 1) establishing a human ovarian tissue and clinical data base core facility to support the proposed projects and future investigations, 2) identifying genes which are differentially expressed in ovarian cancers and thereby discovering biomarkers for early detection, 3) studying ovarian tumorigenesis in ovarian tissues obtained from germline *BRCA1* mutation carrier to better understand the interaction between mutational inactivation of *BRCA1*, the cellular caretaker gene and *p53*, the cellular gatekeeper gene, and 4) developing a genetically defined mouse model of epithelial ovarian cancer.

To date, the ovarian tissue core has banked over 160 surgical specimens and provided sufficient resources for the ongoing projects and other collaborative research on ovarian cancer etiology. Representational difference analysis was used to identify 160 genes specific for normal ovarian epithelium and 95 genes specific for ovarian cancer. The *BRCA1*-mutation associated ovarian tissues required to understand the functional interaction between of *p53* and *BRCA1* have been identified. And, a new viral construct carrying the cre recombinase under the control of the K18 promoter has been tested to establish its ability to mediate recombination in mouse ovarian epithelial cells.

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Genetic Definition and Phenotypic Determinants of Human Ovarian Carcinomas

Beth Y. Karlan, MD, Principal Investigator

INTRODUCTION:

It is estimated that 23,400 women will be diagnosed with ovarian cancer in the year 2001, and 13,900 women will succumb to this disease. In recent years, we have seen a slight decline in the number of new cases of ovarian cancer, and survival from ovarian cancer has been prolonged by improvements in surgery and chemotherapy. However, substantial progress towards ultimately eliminating ovarian cancer as a threat to women has been undermined by our ignorance about its etiology. Without additional insight into the genetic alterations that result in the clinical entity of ovarian carcinoma, we are left with empiric approaches to prevention, early detection and therapy. This program project is aimed at approaching the ovarian cancer problem by 1) establishing a human ovarian tissue and clinical data base core facility to allow the successful completion of the proposed projects and future studies aimed at understanding and eradicating ovarian cancer, 2) identifying genes which are differentially expressed in ovarian cancers and using this information to discover biomarkers for early detection, 3) studying ovarian tumorigenesis in "high risk" ovarian tissues obtained from carriers of germline *BRCA1* mutations to better understand the interaction between mutational inactivation of *BRCA1*, the cellular caretaker gene and *p53*, the cellular gatekeeper gene, and 4) developing a genetically defined mouse model of epithelial ovarian cancer which would be a vital tool for further studies of ovarian cancer etiology, prevention and therapy. This annual report prepared for the USAMRMC describes our progress towards achieving these goals during our first two years of funding.

BODY:

Ovarian Tissue and Clinical Database Core Facility
Beth Y. Karlan, MD, Principal Investigator

In the past, established epithelial ovarian cancer cell lines have been used to study the molecular pathophysiology of human ovarian cancer. These cell lines, however, are very distant from their original *in vivo* state. Following many generations of subculture, the subsequent genetic alterations and clonal selection biases that ensue make cell lines an imperfect experimental system for ovarian cancer. To address this problem, we have developed a human ovarian tissue resource and created a core facility to provide the necessary human ovarian tissues and cellular reagents required to successfully complete the three projects contained in this proposal.

In December 2000, the core facility moved to it's current location in the new wing of the Davis Research Building. This move was accomplished with virtually no down time or impact on our accomplishments. The core facility now occupies rooms 1094C and 1095. This was a positive action. The new laboratories are designed based on state-of-the-art architectural design for research making them very efficient. In addition, we are now physically located in closer proximity to the operating suite than at our previous location. Our new location in the Davis Research Building puts the Core facility in closer proximity to many research laboratories which will foster scientific interactions and collaborations.

TASK 1: Provide a continuing resource of normal and carcinomatous ovarian tissues from patients with benign gynecologic conditions and sporadic and familial ovarian cancer, respectively (months 1-36):

The Core Facility continues to provide all requisite ovarian tissues for the proposed projects. These have included: 15 snap frozen tumor specimens and 7 cell pellets from ovarian cancer ascites to Project 1, as well as multiple histopathology cut sections from prophylactic oophorectomy specimens removed from 7 *BRCA* mutation carriers to Project 2. A significant number of primary ovarian cultures were also provided, as outlined under Task 2. In addition, during the first two years of funding, the Core Facility has banked 167 surgical specimens, including 96 ovarian carcinomas and 71 benign ovaries. Of the 71 benign ovaries, at least 30 were donated from patients with a family history of ovarian and/or known *BRCA1* or *BRCA2* germline mutations. We also obtained matching germline DNA and serum samples from all patients donating surgical specimens. As well as sera from "non'donating" consenting patients (women who had consented to the protocol pre-operatively, but who had insufficient tissue for research donation).

In year one of funding, our progress establishing ties with neighboring and international medical centers was hindered by new regulations implemented by all participating Institutional Review Boards for protecting the rights of human subjects involved in research. These new regulations, designed to provide better protection to human research subjects, include new and detailed applications, separate Genetic Research applications, a new detailed consent form style and a training requirement for all investigators on the inclusion of human subjects in research. Drs Beth Karlan and Rae Lynn Baldwin completed the required training and we have acquired IRB approval from UCLA Medical Center for the banking of surgical specimen and blood samples and we have accrued our first patient from UCLA. All contacts and procedures have been established and we expect to continue patient accrual from this external site. Establishing ties with international medical centers is proving to be more difficult than with regional and national centers, we will pursue institutions outside the U.S.

- Determine *BRCA* mutation status on consenting ovarian cancer patients and screening program participants. Store and highlight these DNA and ovarian samples (when available) (months 1-36).

Genomic DNA samples from 69 consenting Jewish ovarian cancer patients was sent to our collaborators in Canada for *BRCA* mutation screening during this funding period. *BRCA1* and *BRCA2* mutation status has been recorded in the Core's Clinical Database.

- Disseminate announcement of the Core Facility's services to the scientific community in Southern California and provide ovarian tissues to approved projects (months 24-46)

The availability of services and resources from the Ovarian Tissue and Clinical Database Core Facility has been disseminated to investigators nationally and in Southern California via oral communication and through recognition of the Core Facility and DOD funding at scientific meetings and on research publications. After reviewing requests, we have provided resources to the three following investigators. William Wilcox, MD, PhD, Department of Medical Genetics, Cedars-Sinai and UCLA Medical Centers, 5 tumor tissue specimen, 10 CSOC and 5 HOSE cultures, FGFR3 expression and splice variants in ovarian cancer; Carolyn Muller, MD, Univ. Of Texas Southwestern Medical School, Dallas, TX, 10 HOSE and 9 CSOC cultures, RASSF1 gene family expression in ovarian cancer; S. Diane Yamada, MD, University of Chicago, Chicago, IL, 10 HOSE cell protein lysates, Evaluation of MKK4 as metastasis suppressor gene in ovarian cancer.

TASK 2: Establish, characterize and maintain monolayer cultures of human ovarian epithelial cells, human ovarian stromal cells and human ovarian carcinomas according to established procedures (months 1-36).

We have provided the requisite ovarian epithelial cell cultures for the projects. Including: 29 CSOC cultures and 13 HOSE cultures to Project 1 during the first 24 months. In addition, of the 45 CSOC cultures established during this time, we have frozen cultures on 21. Last year we had a higher than usual attrition rate due to fungal infections. These infections were due to construction in and around our tissue culture facility which has resulted in water leaks and other damage. The construction was completed and we did not suffer in additional infections. Likewise we have not exposed to any adverse conditions in our new location in the Davis Research Building which verifies that our procedures to limit and eliminate infections are. We have also established 30 HOSE cultures of which 19 have been cryopreserved and 28 ovarian stromal cultures of which 17 have been frozen.

TASK 3: Expand and maintain the clinical database to serve as an ongoing resource for translational studies (months 1-36).

Clinical, epidemiologic and demographic data as well as specimen inventory information is now being entered as samples are collected and banked. We are actively formatting historical data on previously banked specimen for downloading and enhancing our reporting capabilities. The major accomplishments for of downloading historical data include:

1. *BRCA1* and *BRCA2* mutation screening results from 300+ women screened through the Gilda Radner ovarian cancer screening program

2. CA125 serum level test results from all 1200+ participants of the Gilda Radner program

3. Inventory and sample site details of all tissues present in the frozen tissue bank

Currently we are finalizing the historic data load for cell culture identification, links to clinical specimens, immunohistochemical characteristics, growth patterns and inventory records of viably frozen cell cultures. The compilation of this historical data into spreadsheet formats compatible with data transfer to the Oracle-based database has been a major undertaking that is all ready giving productive results for research design and data acquisition and analysis.

We have also made many major and minor enhancements to improve and expand the utility of the database. Some of the enhancements made to the Database during this funding period include:

1. Added new field to store medical record numbers from patient outside of Cedars-Sinai Medical Center

2. Added an additional tab to the laboratory section for the collection and establishment of lymphoblastoid cell lines from patient peripheral blood

3. Added an additional new tab to the laboratory section for the collection and testing of plasma specimen

We have also implemented data query and analysis software using the database platform. We are using the web-based version of BrioQuery Explorer for free form data query design and formatting. Dr. Baldwin and Mike Rousseve, Research Associate completed a training course in this analysis program and are now technically versed in this function and used BrioQuery Explorer to access and evaluate the data used in this annual report.

Project #1, Molecular Biomarkers in Ovarian Cancer
David D. Chang, MD, PhD Principal Investigator

Cancer diagnosis is based on the detection of features that are unique to transformed cells. Each unique phenotype displayed in cancer cells must be accompanied by changes in gene expression. The genes that are differentially expressed in ovarian cancers compared to their normal counterparts therefore constitute logical candidates for molecular biomarkers for cancer detection. In Project 1, we proposed to conduct a detailed analysis of gene expression differences underlying human ovarian carcinogenesis and use the information to develop biomarkers for ovarian cancer.

TASK 1: To clone genes that are differentially expressed in ovarian cancer and determine their expression profile (months 1-12).

The main objective of our project is to study the gene expression differences underlying human ovarian carcinogenesis. As proposed in Task 1 of the original application, we have conducted a representational difference analysis (RDA) using primary cultures of normal human ovarian surface epithelium (HOSE) and Cedars-Sinai ovarian carcinoma (CSOC). The rational for using cultured ovarian epithelial cells for gene expression analysis was based on the fact that the epithelial cells, which give rise to ~90% of ovarian cancer, constitute a very small fraction (<1%) of the total ovarian mass. We hypothesized that using primary cultures of normal and malignant ovarian epithelium for differential gene expression analysis would preferentially identify epithelial cell-specific genes. We have successfully identified 160 HOSE-specific and 95 CSOC-specific genes from our initial analysis, which employed HOSE and CSOC cultures obtained from two different patients. The expression of these cloned genes were surveyed in 5 additional HOSE and 10 additional CSOC cultures to identify 46 HOSE-specific and 14 CSOC-specific genes that exhibited at least 2.5-fold

difference in expression level. Based on this encouraging result, we expanded our differential gene expression analysis into a gene expression profiling study. Using oligonucleotide-based microarray chips capable of assessing expression of >12,000 genes, we examined the gene expression patterns in 10 HOSE and 22 CSOC samples.

The gene expression profiling studies revealed several promising targets that could be further pursued in biomarker studies. Included in the list of genes we are further charactering are periostin, transglutaminase-2, several protease inhibitors, and PDGF receptor. We have generate a high titre anti-periostin antibodies, which has been used to detect the presence of periostin in ovarian cancer samples by immunoblot and immunohistochemistry.

TASK 2: To characterize the protein coding information and subcellular localization of the differentially expressed genes (month 9-24).

In parallel to the above gene expression studies, we have started biochemical studies on selected genes as proposed in Task 2. In particular we have characterized a gene known as periostin (or osteoblast specific factor 2). Pertinent findings from this analysis include: (I) periostin is a secreted protein; (ii) malignant ascitic fluid contains high amount of periotin; (iii) periostin mediates avb3 and avb5 integrin dependent cell adhesion and migration of ovarian epithelial cells. We plan to extend this line of analysis by examining whether periostin promotes intraperitoneal spread of ovarian cancer cells in year 2.

TASK 3: To study the utility of differentially expressed genes as molecular biomarkers for ovarian cancer (months 21-36).

Not accomplished at this time.

Project #2, Interactions Between *BRCA1* and *P53* Mutations in Human Epithelial Ovarian Carcinogenesis
Mark D. Pegram, MD, Principal Investigator

The molecular/genetic alterations responsible for the genesis of epithelial ovarian cancer are poorly understood. Recent molecular epidemiologic studies have defined a role for the *BRCA1* tumor suppressor gene in familial breast and ovarian cancer syndromes. Using a full-length sequencing strategy, we have recently identified a high incidence (62/108) of *p53* tumor suppressor gene mutations in sporadic epithelial ovarian cancers. This finding has recently been confirmed in our screen for research subjects in a prospective randomized trial of *p53* adenovirus gene therapy for

newly diagnosed ovarian cancers in which ~70% of screened subjects had sequence confirmed *p53* mutations (which was an eligibility criteria for this clinical trial).

Several lines of experimental evidence suggest that *BRCA1* and *p53* may act in concert in DNA damage response and repair pathways: 1) both *p53* and *BRCA1* are physically altered in response to DNA damage, *p53* by stabilization and *BRCA1* by hyperphosphorylation and nuclear relocalization, 2) *p53* and *BRCA1* molecules have a direct physical interaction, 3) both *p53* and *BRCA1* activate P21/WAF-1/cip1 as a common target gene, 4) *BRCA1* is a transcriptional co-activator of *p53*, and 5) early embryonic lethality in *BRCA1* knock-out mice is partially rescued by mutation of *p53*. Furthermore a very high percentage of breast cancers with *BRCA1* mutations exhibit *p53* mutation. We hypothesize that familial ovarian cancer tumorigenesis caused by mutational inactivation and allelic loss of the cellular caretaker gene *BRCA1 requires* the mutational or functional inactivation of the cellular gatekeeper gene *p53* which controls cell cycle checkpoints and/or directs cells to undergo apoptosis. If our hypothesis is correct then we expect to find mutational or functional inactivation of *p53* in 100% of ovarian cancers from *BRCA1*-affected individuals. To test this hypothesis we are collecting and characterizing *p53* expression levels and mutational status in ovarian cancers from a large cohort of known *BRCA1* mutation carriers. This project is relevant to understanding the etiology of familial ovarian cancer which accounts for 10% of all ovarian cancers and as such represents a significant public health problem. Moreover, greater understanding of the biology of familial ovarian cancer will lead to improved diagnostic techniques which may have the potential to be exploited clinically in the management of patients with this disease. For example, a patient with known germline *BRCA* mutation and evidence of *p53* mutation in preneoplastic ovarian tissue may be at especially high risk for malignancy and prophylactic intervention either with surgery or with participation in biochemical prevention trials may be appropriate. The hypothesis that *p53* tumor suppressor function is required for *BRCA1*-linked ovarian tumorigenesis is testable using the human tissue resources available in our laboratory and the Ovarian Tissue Core Facility. We have made considerable progress on the aims and objectives which we originally proposed for this project. The objectives for Project #2 are outlined as follows:

TASK 1: Identification and histologic analysis of ovarian cancer specimens with sequence-verified germ line *BRCA1* mutations (months 1-36).

This task is relevant to Aim I of the proposal: to assess the frequency of *P53* gene mutations in epithelial ovarian cancers with known mutations of the *BRCA1* gene. During the initial year of funding we identified ~71 malignant ovarian tumors in the Core's ovarian tumor bank for which peripheral blood lymphocyte DNA is available and will be tested for *BRCA1* and *BRCA2* mutations. Since that time we have continued to add to this number at the anticipated rate of 10 - 15 cases per year via the ongoing Core Project effort at UCLA and Cedars Sinai Medical Centers. We have been

unable to obtain more samples through our originally proposed collaborative efforts because of new IRB restrictions on identification of human subjects with known germ line molecular genetic alterations. We are thus not allowed to identify potential participants for this study based on tumor bank data registry of known *BRCA* mutational carriers. Therefore we will continue our ongoing effort to collect specimens at our institutions prospectively.

TASK 2: Assessment of frequency of *P53* gene mutations in *BRCA1*-linked ovarian tumors identified in Task 1 above (months 6-36).

Task 2 is also relevant to Aim I of the proposal. In order to assess the frequency of *P53* mutation in *BRCA*-linked ovarian cancer, we first developed DNA sequencing reactions to study mutational frequency. Conditions have been optimized for each of the PCR reactions required for sequencing runs using genomic DNA extracted from tumor tissues obtained from the Core Ovarian Tumor Facility. Now that TASK I is nearing completion, we anticipate being able to complete the proposed sequence analysis during Y3 of the proposal. Batching of samples for sequence analysis all at one time (Y3) is more time efficient that processing samples one at a time as they are being collected. We are currently conducting pilot studies with some of the available tissues to determine whether it will be necessary to microdissect tumor cells from each of the banked ovarian tumor samples in order to avoid missing *p53* mutations due to stromal cell contamination of tumor tissues which would contain wild-type *p53* and could confound results from PCR amplification from genomic DNA extracted from ovarian tumor tissue. Strategically, because of the relatively high frequency of *p53* mutation in this disease, it may be possible to complete sequence analysis of available tumor samples without microdissection, with the need for microdissection being reserved for those cases which are found to be wild-type on the original sequence analysis. Using this strategy, false negative results may be avoided by confirming *p53* mutational status on microdissected tumor cells. Another group has recently reported analysis of *p53* mutational status on *BRCA*-linked ovarian cancers (Buller, et al., Clin Cancer Res. 2001 Apr;7(4):831-8.). These investigators have reported as very high frequency of mutations as we have hypothesized in this proposal. No unique types of *p53* mutations were identified as being specifically associated with a mutant *BRCA* genotype in this study.

TASK 3: Assessment of MDM2 expression in *BRCA1*-linked and sporadic ovarian cancers using immunostaining techniques and compilation of *BRCA1* genotype data from all subjects (months 1-24).

Analysis of MDM2 expression in the available Core Facility Tumor Bank is currently ongoing. In addition we have access to a new large tumor bank from Munich with long-term clinical follow-up. We have recently utilized this cohort to measure expression of UPA and PAI-1 to demonstrate the

prognostic significance of these markers in ovarian cancers of different clinical stages (Konecny, et al., Clin Cancer Res. 2001 Jun;7(6):1743-9). We aim to develop an ELISA format assay suitable for measurement of MDM2 expression using these same samples. Such analysis would expand the number of available samples for analysis significantly.

TASK 4: To determine whether expression of wild-type *P53* in *P53*-mutant, *BRCA1*-mutant CSOC is sufficient to induce cell cycle arrest, induction of *P21* expression, and/or apoptosis (months 6-20).

This task addresses Aim III of the proposed research: to determine whether overexpression of wild-type *P53* is sufficient to induce cell cycle arrest/apoptosis in *BRCA1*-mutant ovarian cells. In this aim, we compare the biological effects of *p53* in *BRCA*-linked ovarian cancer cells to those which are wild-type for *BRCA* genes. Our preliminary data indicate that one of the most important determinant for response to adenoviral vector containing *p53* cDNA is in fact expression of the adenovirus receptor p46 hCAR. However, cells which are *p53* mutant and express hCAR all respond to *p53* transfection in terms of induction of apoptosis, including CSOC lines which are known to harbor *BRCA*-1 mutations. We have now cloned the cDNA for human 46kD hCAR and have developed expression vectors for this cDNA. Transfection of hCAR into hCAR-negative ovarian cancer cells allows further ability to transduce these lines with our *p53* adenoviral vector so that we can now evaluate response to *p53* adenovirus in most all of the available CSOC lines. This finding also may have implications for Project #3 in which adenoviral vectors had been proposed for transfection studies of ovarian surface epithelium *in vivo*.

TASK 5: To determine the frequency of *P53* alterations in "normal" ovaries from *BRCA1*-linked individuals who have undergone prophylactic oophorectomy (months 12-24).

This task addresses Aim II of the proposal: to determine the frequency of *P53* mutations in "normal" ovarian surface epithelium from *BRCA1*-affected individuals undergoing prophylactic oopherectomy. This aim was cited by reviewers as the most difficult to complete. We have recently identified and evaluated normal ovarian tissues from several samples form the Ovarian Tissue Core Facility which met criteria for this study. Using an immunohistochemical screen for aberrant accumulation of mutant *p53* protein, we have sought evidence of *p53* mutation in these otherwise normal tissues. Thus far we have not identified cases of *p53* alteration in the tissues so far evaluated with this methodology. There are at least two limitations to this approach however. One is that ovarian surface epithelium is frequently denuded from the ovarian samples during tissue procurement and processing in the pathology laboratory so it may be difficult to find the cells of interest on the slides following *p53* immunostaining. Moreover, we know from study of malignant ovarian tissue that there can be false negative results from *p53* immunostaining resulting from

truncation mutations of *p53* due to point- or frameshift-mutations resulting in premature stop codons. Therefore it is anticipated that further study of these tissues may be required using microdissection followed by DNA extraction, PCR amplification, and sequence analysis for *p53*.

Project #3, Manipulation of Genes in the Ovarian Epithelium of Mice
Timothy F. Lane, PhD, Principal Investigator

Without models to test ideas about the initial stages of ovarian cancer, the task of identifying relevant markers and relevant targets for therapy becomes a daunting search for a needle in a haystack. One problem is the lack of a genetically defined animal model of epithelial ovarian cancer that can be used to test genes and gene pathways for their involvement during disease development. Techniques have now been developed for introducing genes into mice and expressing them in virtually any tissue type. Such an approach has led to major advances in our understanding of genes involved in a large number of other cancers. Successful demonstration of the utility of such an approach will make it possible to test the involvement of virtually any gene in ovarian cancer progression, and would be a major advance to the field. The strategy we proposed was to test the ability of wild type and modified adenovirus to deliver genes to normal ovarian epithelial cells *in vivo* with the idea that we could then use the cre-lox system to activate or delete genes of interest in a tissue specific fashion.

TASK 1: To establish the efficacy of Ad5-cre delivery to the ovarian epithelium (months 1-24).

The experiments specified in Task 1 of our Statement of work are progressing nicely. As specified in Task 1, we used a recombinationally activated gene cassette that would allow the production of a β-galactosidase gene only in cells expressing cre recombinase, this cassette is referred to as RABE. Several experiments have been carried out *in vitro* showing that the components of the system work well in cultured primary epithelial cells, and this allowed us to move into work on RABE transgenic mice. To date, we have injected Ad-cre into the ovarian capsule of 4 RABE female mice. We have looked for expression of β-galactosidase at 8 hrs (2 mice) and 24 hrs (2 mice). β-galactosidase positive cells were identified only in the 24 hr time point and transduction appeared to be rather inefficient. We are currently producing more concentrated viral stocks in the hopes that poor infection rates can also be overcome with higher titers.

A complication was also quite evident. Adenoviral injections resulted in recruitment of a lymphoid infiltrate into sites of injection. We may request additional funds to document the cell types involved, but will likely switch to our proposed alternative strategy of transplanting the ovaries to avoid the need for exposure of animals to large amounts of virus.

We are also generating more mice to try the alternative strategy proposed for infecting the cells (ovarian transplants). This has been delayed because some of the mice became contaminated with a murine virus (MPV) and had to be destroyed. In year 2, we reestablished a clean colony of RABE mice, but continued too have problems with the RABE transgenic line due to MPV infection and eventually decided to discontinue the line. In to replace the line we have obtained a ROSA26 (129s-gtrosa 26) from Jackson Labs which should be suitable. These mice are obtained in July and the colony has now been expanded to a useable size for our experiments. This has been a setback, but we hope to rapidly progress through the studies that were started in year one. Specifically, we have learned that direct injection of Ad5 particles is impractical due to immune infiltrate into the peritoneal cavity. and hope to continue the experiments by mid October.

TASK 2: To establish the ability of Ad5-cre delivery to delete genes from the ovarian epithelium (months 1-28).

The experiments specified in Task 2 are currently behind schedule due to a backlog in the availability of floxPTEN mice from Dr. Wu. As with our RAGE mice, the floxPTEN colony was dealt a set back from MPV infection. Thus we will be focusing our efforts on the more time consuming alternative strategy of infecting ovaries dissected from transgenic donors and then reimplanting them into host females. We have tried several pilots of this strategy and have encountered the following difficulties: i) it is very difficult to manipulate mouse ovaries without removing the ovarian epithelium. ii). we were not able to use our standard FVB or 129SvEv mice as recipients of ROSA ovarian transplants due to tissue rejection. These difficulties were not unexpected and will be overcome through use of syngeneic ROSA129 hosts and improved skill in transplantation.

TASK 3: To establish the ability of Ad5-cre delivery to activate over-expression of transforming oncogenes in the ovarian epithelium (months 12-36)

TASK 4: To establish the ability of Ad5-cre delivery to activate over-expression of dominant negative anti-oncogenes in the ovarian epithelium (months 24-36).

The experiments specified in Tasks 3-4 rely on development of plasmid vectors and transgenic mice in Task 3. The RA-her2 vector is currently near completion and will be tested *in vitro* ahead of schedule. Establishment of RA-DN*p53* animals are scheduled to start at the end of November, 2001, and the RA-herB2 2will be injected in January 2002. Constructs for the *p53* were delayed because of the MPV infection in our colony. This infection has now been eliminated and we are scheduling all transgenic injections to proceed very rapidly.

KEY RESEARCH ACCOMPLISHMENTS:

Ovarian Tissue and Clinical Database Core Facility

- Collected and snap froze surgical specimen from 96 Ovarian cancer patients
- Collected and snap froze surgical specimen from 71 patients with benign ovarian of which 16 were from patients with family history of ovarian cancer or *BRCA1* or *BRCA2* heterozygotes.
- Collected blood and isolated serum and genomic DNA from all patients that donated surgical tissues.
- Established primary cultures from 45 malignant ovarian tumors and 30 normal ovaries
- Cryopreserved primary cultures from 21 malignant ovarian tumors and 19 normal ovaries
- Provided all material requested to the 3 projects outlined in the Genetic Definition and Phenotypic Determinants of Human Ovarian Carcinomas Project
- Provided resources to the ovarian cancer scientific community
- Expanded and enhanced the Ovarian Tissue and Clinical Database Core Facility electronic database

Project #1, Molecular Biomarkers in Ovarian Cancer

- Cloned genes that are differentially expressed in ovarian cancer cells using cDNA-RDA.
- Sequenced cloned DNA fragments to identify 160 HOSE and 95 CSOC specific genes.
- The cloned DNA fragments were used to fabricate a high density DNA arrays. These arrays were interrogated with cDNA probes from 15 different HOSE and CSOC cells to identify 46 HOSE-specific and 14 CSOC-specific genes that exhibited at least 2.5-fold difference in expression level.
- Constructed an ovarian epithelial cell cDNA library.
- We carried out a gene-expression profiling study using 10 HOSE and 22 CSOC samples
- We have generated a high quality anti-periostin antisera to study the expression of periostin in ascites and sera.
- We have established immortalized ovarian epithelial cell lines by transducing the catalytic subunit of telormerase (hTERT) and/or HPV-16 E7.

Project #2, Interactions Between *BRCA1* and *P53* Mutations in Human Epithelial Ovarian Carcinogenesis

- Identified *BRCA1*-linked ovarian cancers in the Ovarian Tissue Core Facility
- Established optimal conditions for each of 10 PCR reactions required for amplification of genomic *P53* sequence. We have confirmed the validity of this approach by sequencing *P53* from MDA-MB-231 cells which are known to harbor a specific *P53* mutation.
- Identified a large cohort of both sporadic and *BRCA*-linked ovarian tumors suitable for MDM2 expression analysis.
- Discovered an association between expression of the human coxsackie and adenovirus receptor and adenoviral vector transduction efficiency in ovarian cell cultures.
- Elucidated anti-adenovirus antibodies as an inhibitor of adenoviral vector transduction in human malignant ovarian cancer ascites specimens.
- Procured the first 8 samples of "normal ovaries" from known *BRCA1* mutation carriers. We will seek evidence of *P53* gene mutation in these premalignant specimens.

Project #3, Manipulation of Genes in the Ovarian Epithelium of Mice

- Created a new construc that carries the *cre* recombinase under the control of the k18 promoter.
- Tested the ability of this construct to mediate recombination in mouse ovarian epithelial cells in culture.
- Generated Ad5-cre under the control of standard adenoviral promoter elements for pilot work *in vitro* and *in vivo*.

REPORTABLE OUTCOMES:

Ovarian Tissue and Clinical Database Core Facility

1. A well endowed human tissue and serum repository for normal and malignant ovarian tissues and corresponding serum and germline DNA.

2. The development and preservation of primary cultures of normal and malignant human ovarian epithelial and stromal cells.

3. The Ovarian Cancer Laboratory and Clinical Database link laboratory specimens and results with patient demographic, epidemiologic and clinical data.

Project #1, Molecular Biomarkers in Ovarian Cancer

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2. Matei D, Graeber T, Karlan BY, and Chang DD: Gene-expression profiling in normal and malignant ovarian epithelia. Submitted: Cancer Research.

Project #2, Interactions Between *BRCA1* and *P53* Mutations in Human Epithelial Ovarian Carcinogenesis

1. Elkas JC, Pegram MD, Nielsen L, Tseng Y, Baldwin RL, Slamon DJ and Karlan BY: Immunoglobulins in malignant ascites inhibit adenoviral infection of tumor cells: Implications for adenoviral gene therapy. Submitted: Human Gene Therapy.

2. Buller RE, Slamon D, Runnebaum IB, Horowitz JA, Buekers T, Salko T, Petrauskas S, Shahin M, Kreienberg R, Karlan B, Pegram M: A phase I/II trial of rAD/*p53* (SCH 58500) gene replacement in recurrent ovarian cancer. Submitted: Journal of Clinical Oncology.

3. Konecny G, Untch M, Pihan A, Kimmig R, Gropp M, Stieber P, Hepp H, Slamon D, Pegram M: Association of urokinase-type plasminogen activator and its inhibitor with disease progression and prognosis in ovarian cancer. Clin Cancer Res, 7(6):1743-1749; 2001.

Project #3, Manipulation of Genes in the Ovarian Epithelium of Mice

1. Developed an adenoviral vector expression *cre* recombinase and a variant with the K18 promoter.

CONCLUSIONS:

This program project is a multidisciplinary collaboration aimed at elucidating genetic alterations that contribute to human ovarian carcinoma with an eye towards identifying useful targets for early ovarian cancer detection and prevention. Towards these ends, the **ovarian tissue core facility** has banked over 160 surgical specimens, including 96 ovarian carcinomas and 71 benign ovaries, of which approximately one third are from women with a family history of ovarian cancer of or a known *BRCA* mutation. Furthermore, the Core's tissue resources are linked to clinical, demographic, and epidemiologic data that allows us to make clinical correlations with our laboratory findings. The Core together with the Clinical Database will support all avenues of ovarian cancer research including those directed toward understanding the basic biology, etiology, genetic influences, prevention and therapeutic developments. In **Project 1**, representational difference analysis was used to identify 46 genes significantly overexpressed in normal ovarian epithelium and 14 specific genes overexpressed in ovarian cancer cells. Using these subtractive cloning techniques as well as a chip based expression profiling approach, we have successfully identified target genes that could be developed into biomarkers for ovarian cancer. In addition, the newly established immortalized ovarian epithelial cell lines will be used to study the functions of the genes that are differentially expressed in cancer-derived ovarian epithelial cells. **Project 2** has established the necessary techniques and identified the *BRCA1* mutation associated ovarian tissues required to understand the functional interaction and contribution of *p53* and *BRCA1* to ovarian epithelial transformation. In addition, as a byproduct of these studies, we discovered that adenoviral gene transfer may only be efficiently accomplished in ovarian cells which express a gene called hCAR (human coxsackie and adenovirus receptor). This observation may have far reaching implications for patients undergoing gene therapy for ovarian cancer using adenoviral vectors. **Project 3** has focused efforts at creating the necessary viral constructs for the proposed experiments aimed at establishing a murine human ovarian cancer model. A new construct carrying the cre recombinase under the control of the K18 promoter has been tested to establish its ability to mediate recombination in mouse ovarian epithelial cells. Work on general application of adenovirus into various mouse tissues has demonstrated complications. We will focus future efforts on using our ability to transplant ovaries to overcome this problem.

"So what?" In order to reduce the unacceptably high mortality rate associated with ovarian cancer, diagnostic modalities which can reliably detect early stage ovarian cancer and preventative strategies to diminish the number of new cases must be discovered. This Program Project has undertaken a multi-faceted approach to the ovarian cancer problem. Using the human ovarian specimens and clinical correlates provided by the core facility, new genes will be identified in Project 1 to serve as targets for detection, prevention, and/or therapy; functional interactions between important known genes, *BRCA1* and *p53*, will be elucidated in Project 2 and shed light on the molecular etiology of

ovarian cancer; and a murine animal model to test these findings and others *in vivo* will be established in Project 3. At the conclusion of this program project, we will be closer to our goal of rationale rather than empiric approaches to ovarian cancer prevention, early detection, and therapy.

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None

APPENDICES

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Differential Gene Expression between Normal and Tumor-derived Ovarian Epithelial Cells[1]

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ABSTRACT

The majority of ovarian tumors arise from the transformation of the ovarian surface epithelial cells, a single layer of cells surrounding the ovary. To identify genes that may contribute to the malignant phenotype of ovarian cancers, cDNA representational difference analysis was used to compare expressed genes in primary cultures of normal human ovarian surface epithelium (HOSE) and ovarian tumor-derived epithelial cells from the Cedars-Sinai Ovarian Cancer (CSOC) repository. A total of 255 differentially expressed genes were identified, of which 160 and 95 were specifically expressed in HOSE and CSOC cells, respectively. Using cDNA array hybridization, the expression profiles of the genes identified by cDNA-representational difference analysis were examined in an additional 5 HOSE and 10 CSOC lines. The comparison of average signal of each gene revealed 44 HOSE-specific and 16 CSOC-specific genes that exhibited at least a 2.5-fold difference in expression. A large number of genes identified in this study encode membrane-associated or secreted proteins and, hence, may be useful as targets in the development of serum-based diagnostic markers for ovarian cancer. Very few genes associated with protein synthesis or metabolism were identified in this study, reflecting the lack of observable differences in phenotypic or growth characteristics between HOSE and CSOC cells. Northern blot analysis on a subset of these genes demonstrated comparable levels of gene expression as observed in the cDNA array hybridization.

INTRODUCTION

Ovarian cancer is the fourth leading cause of cancer-related deaths in the United States (1). Each year, ~23,000 women will be diagnosed with this disease and close to 14,000 will die from it. A significant factor contributing to the high mortality rate of ovarian cancer is the relatively asymptomatic progression of this disease. As a consequence, most patients are diagnosed with advanced (stage III/IV) disease when widespread i.p. metastases are already present (2). Greater than 90% of ovarian malignancies arise from the transformation of the ovarian surface epithelium, a single continuous layer of epithelial cells surrounding the ovary.

There are estimated to be 20,000 genes expressed in a typical cell, and ~1% of those is differentially expressed in cancerous *versus* normal cells (3). A limited number of genes has been found to have elevated or depressed levels of expression in ovarian cancers when compared with normal tissue (4–8). As in other neoplasm, it is generally accepted that both activation of oncogenes and inactivation of tumor suppressor genes are involved in the etiology of ovarian carcinomas. Brca1, Brca2, and p53 mutations are associated with the development and progression of ovarian cancer (9–11). Because these proteins are involved in the maintenance of genomic integrity, loss of their functions is thought to result in the accumulation of genetic mutations, leading to extensive changes in gene expression (12–15). Comparison between gene expression profiles of normal ovarian epithelial cells and ovarian tumors could identify candidate genes for biological markers of cellular transformation, possibly leading to earlier detection and new therapy.

Because ovarian epithelial cells represent a small proportion of the total cells found in the normal ovary, it is difficult to obtain primary material that is free of contaminating ovarian stromal cells in large enough quantities to conduct comparative gene expression studies. However, ovarian epithelial cells can be isolated and expanded in culture for ~15 passages (16, 17). The ability to culture human ovarian epithelial cells from both normal ovaries and ovarian carcinomas provides an opportunity to study differential gene expression between relatively pure populations of normal *versus* tumor-derived epithelial cells. This type of comparison minimizes gene expression differences that reflect the presence of nonepithelial cells, such as stromal or germ cells of normal ovaries and host-derived immune cells in ovarian tumors.

We used cDNA-RDA[3] to identify a set of genes that are differentially expressed between primary cultures of normal and tumor-derived ovarian epithelial cells. cDNA-RDA was subsequently combined with cDNA filter array hybridization to identify a subset of genes that are aberrantly expressed in a large number of malignant ovarian epithelial cells. Direct gene expression profiles were obtained by Northern blot analysis on four differentially expressed genes to confirm the cDNA array analysis.

MATERIALS AND METHODS

Primary Ovarian Epithelial Cell Cultures. The conditions for growing normal HOSE cells *in vitro* were modifications of the method described by Auersperg *et al.* (18). Briefly, normal ovarian tissue was obtained from the operating room from consenting donors and placed in 199:MCDB 105 (1:1) medium (Sigma Chemical Co., St. Louis, MO) containing 10% FCS, 200 u/ml penicillin, and 200 μg/ml streptomycin. Epithelial cells were microdissected or scraped from the ovarian surface. The epithelial explants were placed in culture medium and allowed to attach and proliferate. Once the epithelial cells reached confluency, the explants were removed and the cells were subcultured. CSOC cultures were established from ovarian carcinomas in a similar manner. Fresh tumor tissue was finely minced with scissors and allowed to attach to culture dishes in McCoy's 5A medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS and penicillin/streptomycin. The epithelial nature of HOSE and CSOC cultures was verified by immunohistochemical analysis with antibodies against cytokeratin (AE1/AE3; Roche, Indianapolis, IN), vimentin (clone v9; Roche), and factor VIII (Factor VIIIC; Calbiochem), as described previously (19). p53 status of cultures was determined by immunostaining with Ab-6 antibody (Roche).

Cell Lines. TfxH, an SV40 Large T antigen-immortalized HOSE cell line, was grown in 199:MCDB 105 (1:1) medium containing 10% FCS (17).

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[3] The abbreviations used are: cDNA-RDA, cDNA representational differences analysis; HOSE, human ovarian surface epithelial; CSOC, Cedars-Sinai Ovarian Cancer; EF-Tu, translation elongation factor; Cx43, connexin 43; OSF-2, osteoblast-specific factor 2; KRT19, keratin 19; STC, stanniocalcin; GDN, glia-derived nexin.

Ovarian carcinoma-derived cell lines, Caov-3 and Sk-OV-3 (American Type Culture Collection, Manassas, VA), were grown in the presence of 10% FCS in DMEM or McCoy's 5A medium, respectively.

Cloning of Differentially Expressed Genes Using cDNA-RDA. cDNA-RDA was used to compare gene expression between two HOSE and two CSOC cultures (20). Total RNA was prepared from each culture, using RNA STAT-60 reagent (Tel-Test, Inc., Friendswood, TX). mRNA was purified from 120 μg of total RNA using Oligotex mRNA columns (Qiagen, Inc., Chatsworth, CA) and used for cDNA synthesis. cDNA from HOSE and CSOC samples was digested with Dpn II and PCR-amplified following the addition of RDA adapters. Subtractive hybridization was performed in 2.5 μl of 3× EEP buffer [10 mM EPPS[*N*-(2-hydroxyethyl) piperazine-N'-3-propanesulfonic acid], 1 mM EDTA, 1 M NaCl, and 10% polyethylene glycol] for 21 h at 67°C. Two rounds of subtraction were performed in both directions, using tester to driver ratios of 1:100 and 1:500 for the first and second rounds, respectively. Finally, the cDNA-RDA products were digested with Dpn II and cloned into the *Bam*HI site of pBluescript KS$^+$ (Stratagene, La Jolla, CA).

Amplification of Individual cDNAs. Individual bacterial transformants were isolated into 96-well microtiter plates containing 100 μl of LB-ampicillin (100 μg/ml) and incubated overnight at 37°C. Using a 96-well replicating tool (V&P Scientific, San Diego, CA), bacterial culture was transferred into 96-well thermowell plates (Costar, Cambridge, MA) to inoculate 50-μl PCR reactions containing 250 μM dNTPs, 1 ng/μl SK (GGCCGCTCTAGAACTAGTGGATC) and KS (TGATATCGAATTCCTGCAGCCCG) primer each, and 0.03 u/μl Taq polymerase (Qiagen, Inc.). Amplification was carried out for 35 cycles (94°C for 45 s, 68°C for 45 s, 72°C for 1 min), with a final 10-min extension at 72°C. The average size of the PCR-amplified fragments was ~500 bp.

Identification of Nonredundant Clones. The PCR-amplified inserts were spotted onto 7.8 × 12.3-cm Hybond N+ membrane (Amersham Corp., Arlington Heights, IL), using a 96-well replicating tool. Redundant clones were eliminated by back hybridization, as described previously (21). The DNA sequences of nonredundant clones were determined using a commercially available sequencing kit (ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit; Perkin-Elmer Corp., Foster City, CA). The nucleotide sequences were analyzed using the BLASTN program and the GenBank database.

cDNA Filter Hybridization of Arrayed Nonredundant Clones. Nonredundant cDNA-RDA fragments were organized into 96-well plates and subsequently arrayed in duplicate on nylon membranes. cDNA array hybridization was carried out as described previously (21). To generate probes, ~50 μg of total RNA from each of 5 HOSE and 10 CSOC cultures were poly(A) selected and used to synthesize cDNA. One-fifth of the total cDNA obtained was labeled with [^{32}P]-α-dCTP, using a random primer labeling kit (Prime-it II; Stratagene). The hybridization signal was quantified on a phosphorimager (Molecular Dynamics), using the ImageQuaNT software package.

Standardization of Quantitative Data. The signal intensity per pixel within each square of the grid was calculated and corrected for the background. Each filter was also spotted with actin, tubulin, glyceraldehyde-3-phosphate dehyrdrogenase and EF-Tu cDNAs. These genes function in housekeeping activities in the cell and display little variability in expression between normal and transformed cells (22). EF-Tu, which showed the least variability in mRNA expression between all of the samples, was used as the internal standard in this study. The signal of each background-corrected spot was determined relative to average background-corrected signal for EF-Tu within a given filter, and averages of HOSE and CSOC standardized signal were compared for each of the 864 dots on the filter. In this study, only those with differences >2.5-fold were considered to be differentially expressed.

Northern Blot Analysis. Total RNA (5 μg) was separated on 0.9% agarose formaldehyde gels and transferred to Hybond-N+ nylon membranes. Filters were hybridized with ^{32}P-cDNA probes corresponding to the differentially expressed genes, as described previously (21). The filters were then stripped and rehybridized with ^{32}P-labeled EF-Tu cDNA to control for mRNA loading.

RESULTS

Ovarian Epithelial Cells Can Be Expanded *in Vitro*. Ovarian epithelial cells tend to assume atypical fibroblast-like morphology and a dual epithelio-mesenchymal phenotype that is characterized by the expression of both keratin (an epithelial marker) and vimentin (a mesenchymal marker) in culture (16). Cultures containing endothelial cells, identified by Factor VIII staining, and ovarian stromal cells, characterized by the lack of cytokeratin staining and low levels of vimentin staining, were excluded in this study. The staining patterns for HOSE and CSOC cells used in the cDNA-RDA and cDNA filter array hybridization are presented in Table 1.

There are no established molecular criteria to distinguish HOSE from CSOC cells. Therefore, we relied on cytogenetic studies to confirm that HOSE and CSOC cultures represent normal and malignant ovarian epithelial cells, respectively. Both HOSE cultures used in the cDNA-RDA contained normal female diploid cells; however, the cells from both CSOC cultures displayed an abnormal karyotype (Table 1). Sixty percent of CSOC 817 cells exhibited a loss of the X chromosome, which is commonly associated with ovarian carcinomas

Table 1 *Characteristics of ovarian epithelial cells used for comparative gene expression studies*

Pathological diagnosis of the primary cultures used for cDNA-RDA or cDNA filter array hybridization experiments are shown. Immunohistochemical analysis was performed to ensure that cells had homogeneous cytokeratin and vimentin staining with no factor VIII staining. p53 immunostaining results are also presented. Increasing intensities of staining are indicated from "+" to "+++".

Culture type	Pathologic diagnosis	Cytokeratin	Vimentin	Factor VIII	p53	Karyotypes	Application
HOSE 231	Normal	++	++	Neg[a]	N/D	46:XX	cDNA-RDA
HOSE 250	Normal	++	++	Neg	N/D	46:XX	cDNA-RDA
CSOC 817	Papillary serous	++	++	Neg	++	45:X[12]/46XX[18] (60% of cells had one X)	cDNA-RDA
CSOC 826	Papillary serous	++	++	Neg	++	47:XX, +mar[2]/46:XX[18] (10% + marker)	cDNA-RDA
HOSE 224	Normal	++	++	Neg	N/D	N/D	cDNA filter array hybridization
HOSE 246	Normal	++	++	Neg	Neg	N/D	cDNA filter array hybridization
HOSE 253	Normal	++	++	Neg	N/D	N/D	cDNA filter array hybridization
HOSE 254	Normal	+	++	Neg	N/D	N/D	cDNA filter array hybridization
HOSE 256	Normal	++	++	Neg	N/D	N/D	cDNA filter array hybridization
CSOC 823	Papillary serous	++	++	Neg	Neg	N/D	cDNA filter array hybridization
CSOC 824	Papillary serous	+	++	Neg	+	N/D	cDNA filter array hybridization
CSOC 827	Papillary serous	++	++	Neg	Neg	N/D	cDNA filter array hybridization
CSOC 834	Papillary serous	++	++	Neg	+	N/D	cDNA filter array hybridization
CSOC 839	Papillary serous	+	++	Neg	Neg	N/D	cDNA filter array hybridization
CSOC 843	Papillary serous	++	++	Neg	++/+++	N/D	cDNA filter array hybridization
CSOC 846	Papillary serous	++	++	Neg	++	N/D	cDNA filter array hybridization
CSOC 869	Papillary serous	+++	++	Neg	++	N/D	cDNA filter array hybridization
CSOC 871	Papillary serous	+++	++	Neg	++	N/D	cDNA filter array hybridization
CSOC 872	Papillary serous	+	++	Neg	+	N/D	cDNA filter array hybridization

[a] Neg, negative; N/D, not determined.

Table 2 *Identification of genes preferentially expressed in HOSE cells*

Genes preferentially expressed in HOSE cells with the GenBank matches are listed. *Bold lettering* indicates genes encoding proteins that are expressed on the cell membrane or that are secreted. Clones that failed to match any entry in the GenBank database (as of November 15, 1999) in the BLASTN searches are considered novel.

2.5–3.0		>3.0	
Name	Accession no.	Name	Accession no.
		Aldehyde dehydrogenase 1	K03000
Adrenomedullin	D14874	**Connexin 43**	**M65188**
BAC 360 F12 Xq28	AC002523	hCOX-2	U04636
Basic fibroblast growth factor	**M27968**	High sulphur keratins-B2A/B2D	X01610
DCRR1	D83327	**ICAM-1**	**J03132**
Fumurase precursor	U59309	Il-6 inducible 26-kDa protein	M54894
PRAME-antigen of melanoma	**U65011**	**Insulin-like growth factor bp 5**	**L27560**
Pyruvate dehydrogenase kinase	U54617	Keratin 19	Y00503
EST-cDNA clone	AA187731	**MASP**	**D28593**
EST-CD44	**N32466**	**MHC DNA**	**AB000879**
EST-gap junctional protein α 5/connexin 40	**AA436946**	Nuclear pore complex-assoc. TPR	U69668
EST-HLA1 MHC27	**AA293071**	**Stanniocalcin**	**U25997**
EST-neuroligin	**AI093247**	EST-BAC clone *3p26-OXTR* gene	AA235188
EST-retanoic acid induced 3 (RAI3)	AA112374	**EST-CD9**	**AA339020**
EST-regulator of G protein signaling (RGS4)	T74284	EST-CTX	AA406389
		EST-DVS27 related protein	AA428482
		EST-human clone 3930	AA776733
		EST-KIAA1199 protein	AA852453
		EST-MHC II α chain	**AA455820**
		EST-pregnancy specific β1 glycoprotein 6 (PSG6)	**AA772680**
EST	N42148	EST	AA425042
EST	AA425042	EST	AA569767
		EST	R18293
2 novel		3 novel	

(23–25). Rearrangements in chromosomes 12 and 18 were also seen in CSOC 817 cells. Ten percent of CSOC 826 cells contained a small chromosome that may be isochromosome 21. A gain in small regions of chromosome 2 was also observed in CSOC 826 cells, which is consistent with a previous study showing frequent gains in discrete regions of chromosome 2 in some ovarian cancer cells (26). These abnormal karyotype profiles of CSOC 817 and CSOC 826 are consistent with the malignant origins of these cultures.

cDNA-RDA Was Used to Identify Differentially Expressed Genes in Ovarian Cancer Cells. A total of 255 nonredundant genes were identified after two rounds of subtractive hybridization. Of these, 95 were preferentially expressed in CSOC cells and 160 in HOSE cells. We then used cDNA array hybridization to identify a subset of genes that were differentially expressed in a larger cohort of HOSE and CSOC cultures. Duplicate filters were spotted with genes identified by cDNA-RDA and hybridized with ^{32}P-labeled cDNA probes derived from additional 5 HOSE and 10 CSOC cultures. Forty-four HOSE-specific and 16 CSOC-specific genes displayed a >2.5-fold difference in expression (Tables 2 and 3). An example of cDNA filter arrays probed with ^{32}P-labeled cDNAs from HOSE 224 or CSOC 869 cells are shown in Fig. 1. cDNA array hybridization easily identified Cx43 and OSF-2 as HOSE- and CSOC-specific genes, respectively. The hybridization signal intensity of Doc-1, which was cloned as a HOSE-specific gene in cDNA-RDA, on the other hand, did not vary significantly between these two HOSE and CSOC cultures. The expression levels of *EF-Tu*, a housekeeping gene shown to be expressed at a constant level in normal and cancer cells (22), remained unchanged in HOSE 224 and CSOC 869 cells.

To assess the predictive value of cDNA array hybridization analysis, four differentially expressed genes were chosen at random and evaluated by Northern blot analysis. *OSF-2* was cloned as a CSOC-specific gene by cDNA-RDA, whereas the other three (*KRT19*, *Cx43*, and *STC*) had been identified as HOSE-specific genes. The expression levels of OSF-2 were greatly elevated in the 2 CSOC samples used for cDNA-RDA, as well as in 6 of the 9 CSOC samples used in array hybridization (Fig. 2). On the other hand, all three HOSE-specific genes were expressed at lower levels in CSOC cells (Fig. 2). For example, there was an overall decrease in Cx43 mRNA expression in CSOC samples compared with HOSE samples. STC and KRT19 were down-regulated in all but two CSOC samples. Interestingly, there was very little correlation in the expression of these genes in established ovarian cell lines. Specifically, OSF-2 was not overexpressed in either of the two established ovarian cancer cell lines Sk-Ov-3 and Caov-3. In addition, Cx43, which was expressed at varying levels in both HOSE and CSOC cultures, was not detected in either ovarian cancer cell lines or an SV40 Large T-antigen immortalized TfxH cell line.

The majority of genes identified by cDNA-RDA was found to be differentially expressed by <2.5-fold in cDNA array hybridization and eliminated from further consideration. To ensure that this arbitrary cutoff level did not eliminate any genes of interest, we performed Northern analysis on two cDNA clones displaying an expression level difference of <2.5-fold (Fig. 3). Northern analysis demonstrated that *Doc-1*, which was identified as a HOSE-specific gene in our study, as well as in a previous study (4), was expressed at higher levels in HOSE samples. However, its expression was variable, accounting for the <2.5-fold difference in cDNA array hybridization, which used average signal intensity in data analysis. *GDN*, a CSOC-specific gene, was up-regulated in both the CSOC 826 and CSOC 817 cells used in the initial cDNA-RDA analysis. In the additional HOSE and CSOC samples, however, GDN was expressed at a variable level in both normal and tumor-derived epithelial cells.

Table 3 *Identification of genes preferentially expressed in CSOC cells*

Genes preferentially expressed in CSOC cells with the GenBank matches are listed. *Bold lettering* indicates genes encoding proteins that are expressed on the cell membrane or that are secreted. Clones that failed to match any entry in the GenBank database (as of November 15, 1999) in the BLASTN searches are considered novel.

2.5–3.0		>3.0	
Name	Accession no.	Name	Accession no.
Collagen α 1 type XV	**L25286**	**Collagen α 2 type I**	**Z74616**
Collagen α 3 type VI	**X52022**	**Osteoblast specific factor-2**	**D13665**
cPLA2	M68874		
E1–E2 ATPase	AF011337	**EST-Annexin IV**	**W12985**
MEGF5	**AB011538**	**EST-annexin VI**	**AA594856**
p37NB	U32907	EST-collagen c-prot enhancer	T49144
SPARC/osteonectin	**J03040**	**EST-collagen**	**Z33436**
EST	R67289	EST	W79345
1 novel			

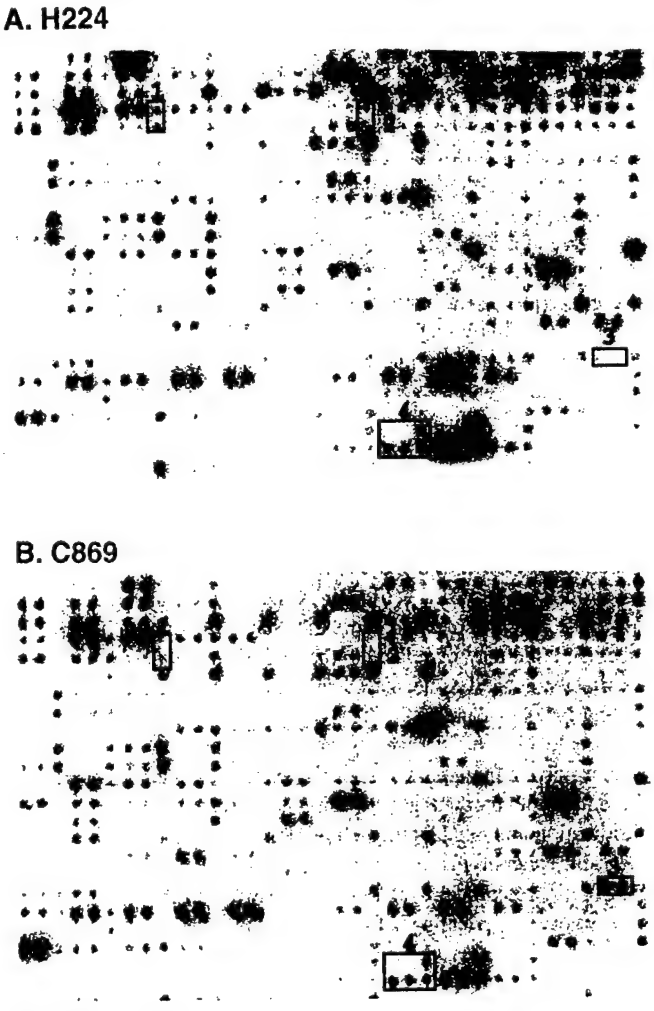

Fig. 1. Analysis of cloned cDNA-RDA fragments by cDNA filter array hybridization. cDNA filter array hybridization was used to screen 255 differentially expressed gene fragments obtained after two rounds of cDNA-RDA. PCR-amplified fragments were spotted in a 96-well format onto nylon filter membranes. Duplicate filters were hybridized with random primed ^{32}P-labeled cDNA probes derived from HOSE culture H224 (*A*) and CSOC culture C869 (*B*). The positions of Cx43 (1), Doc-1 (2), OSF-2 (3), and EF-Tu (4) are *boxed*. The differences in the signal intensities of Cx43 and OSF-2 in H224 and C869 can be visually appreciated. In contrast, the signal intensities of Doc-1 in these two samples were not significantly different. The expression levels of EF-Tu, which remained relatively constant in H224 and C869, were used to standardize the hybridization signal.

OSF-2 Is Overexpressed in Both Ovarian Tumors and *in Vitro* Expanded CSOC Cells. We next studied the expression of OSF-2 in ovarian tumor samples from which the CSOC cells used in this study were derived. Total RNA was isolated from the quick frozen tumor samples corresponding to 6 of the 12 CSOC cultures used in this study. Northern analysis revealed expression of OSF-2 in all but one tumor sample (T949; Fig. 4). The OSF-2 expression was low in the CSOC culture (C824), derived from the same tumor (see Fig. 2*A*). In T1040, the level of OSF-2 expression was lower than the level observed in the corresponding C871, but was easily detectable. Overall, there was a high degree of concordance in the OSF-2 expression in tumors and the corresponding CSOC cultures, indicating that the observed overexpression of OSF-2 in CSOC cultures is not a consequence of *in vitro* expansion of tumor-derived epithelial cells.

DISCUSSION

Identifying genes that are differentially expressed in ovarian tumors when compared with their normal counterpart is a challenging issue because of the scarcity of the normal ovarian epithelial cells. Ovarian surface epithelia from which ovarian cancers originate represent a minute cellular component of the ovary, compared with the more abundantly present stromal cells and germ cells. We used *in vitro* expanded ovarian epithelial cells derived from either normal ovaries or ovarian tumors to identify 60 genes that are either up- or down-regulated in ovarian cancer cells. In this study, only the cancer cells derived from papillary serous histology tumors, which is considered to be the most common histological type of ovarian cancer, were included to limit the complexity of gene expression analysis.

Because of the variability in signal intensities in array hybridization analysis, we focused our attention on genes displaying a >2.5 fold difference in the expression level. Whereas the reliance on cDNA array hybridization with this arbitrary cutoff may have eliminated some differentially expressed genes from our analysis, it was highly effective in identifying genes that display a consistent pattern of expression differences in a large number of CSOC samples. Among the subset of genes displaying an expression level difference of >2.5-fold there was strong qualitative correlation between cDNA array hybridization data and Northern analysis. Northern analysis

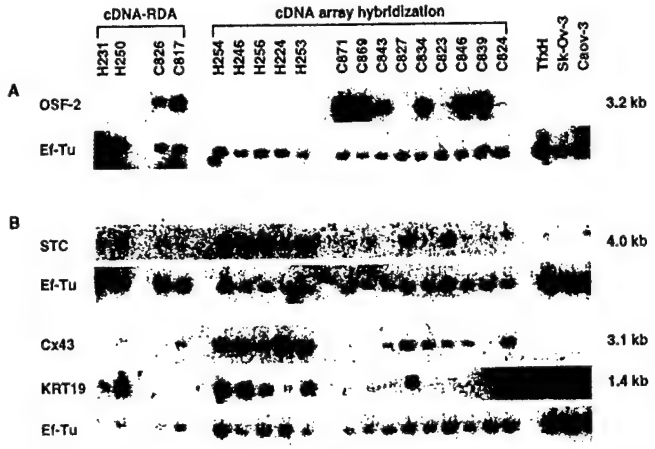

Fig. 2. Northern blot analysis of genes that were differentially expressed by >2.5-fold. RNA prepared from 7 HOSE and 11 CSOC cells used in cDNA-RDA and cDNA array analysis was hybridized with ^{32}P-labeled probes of four randomly chosen genes that were differentially expressed by >2.5-fold in cDNA array analysis. *OSF-2* was cloned as a CSOC-specific gene (*A*), whereas *STC*, *Cx43*, and *KRT19* were cloned as HOSE-specific genes (*B*). In cDNA array analysis, the average signal intensity of OSF-2 was 65-fold greater in CSOC cells, compared with HOSE cells. STC, Cx43, and KRT19 were preferentially expressed in HOSE cells by 7-fold, 2.6-fold, and 7-fold, respectively. The filters were stripped and reprobed with EF-Tu to ensure integrity of RNA and to determine RNA loading differences.

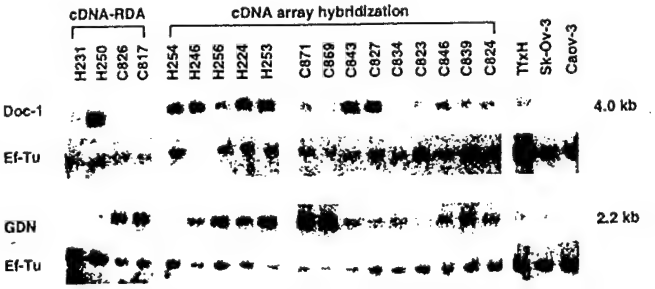

Fig. 3. Northern blot analysis of genes that were differentially expressed by <2.5-fold. RNA prepared from 7 HOSE and 11 CSOC cells used in cDNA-RDA and cDNA array analysis were hybridized with Doc-1 or GDN probes. *Doc-1* and *GDN* were cloned as HOSE- and CSOC-specific genes, respectively. In cDNA array analysis, the average signal intensities of the Doc-1 were 1.6-fold greater in HOSE cells, whereas the signal for GDN was almost even in CSOC and HOSE cells. The same filter membrane was stripped and reprobed with EF-Tu to ensure integrity of RNA and to determine RNA loading differences.

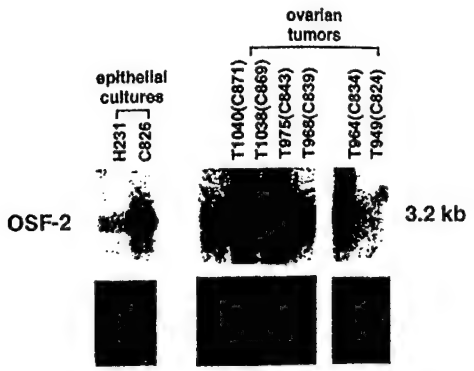

Fig. 4. OSF-2 expression in ovarian tumors. RNA prepared from ovarian tumors was hybridized for OSF-2. The six tumors used in this analysis represent original ovarian tumors from which 6 of the 10 CSOC cultures used in cDNA array analysis were derived. Ethidium bromide staining of rRNA is provided below to control for the integrity and amount of total RNA (5 μg) loaded in each *Lane*.

confirmed *KRT19*, *Cx43*, and *STC* as HOSE-specific genes and *OSF-2* as a CSOC-specific gene. Two genes, *Doc-1* and *GDN*, with expression level differences of <2.5-fold in cDNA array hybridization, on the other hand, displayed only a moderate difference (*e.g.*, GDN) or a high sample-to-sample variability (*e.g.*, Doc-1) in Northern analyses.

The gene expression patterns seen in HOSE and CSOC cells were not consistent with those seen with TfxH cells, an immortalized HOSE cell line, or Caov-3 and Sk-OV-3, two ovarian carcinoma-derived cell lines. Because these cell lines were originally derived from normal epithelial or tumor-derived epithelial cells, similar to HOSE and CSOC cells (27), our results illustrate the divergence of gene expression that can occur as the result of long-term *in vitro* manipulation of these cells. Although cell lines provide a relatively simple model to examine gene expression in ovarian cancer-derived cells, our findings emphasize that the use of HOSE and CSOC cultures represents a better model system of normal and cancerous ovarian tissues in comparative gene expression analysis.

The use of cultured ovarian epithelial cells is not without concerns. Ovarian tumors frequently are histologically inhomogeneous (2). There are reports in the literature of loss of tumor markers associated with continuous tissue culture of some xenograft lines (28–31). Despite the primary tumors containing areas of differentiated cells, in each instance, a selection of a poorly differentiated subpopulation had occurred during the propagation of these lines. Although we have used primary cultures to avoid a selection bias inherent in any long-term cultures, we cannot formally exclude the possibility that our cultured CSOC cells represent a subpopulation of cancer cells present in the original tumor, or *in vitro* expansion conditions may have modified gene expression. In the case of OSF-2, there was a high degree of concordance in its expression in tumors and their corresponding CSOC cells, indicating that despite the *in vitro* expansion process, the expression of this particular gene is preserved in the cultured cells.

The extent of gene expression differences between HOSE and CSOC cultures is not known. Numerous genes identified in this study, including *Doc-1*, have previously been shown to be differentially expressed in ovarian carcinomas (4–8). Comparing our data to previous studies on ovarian cancer-related genes revealed only a minor degree of overlap, indicating that the extent of gene expression differences far exceeds the number of genes identified in this or other previous studies. Genes associated with protein synthesis or mitochondrial metabolism are frequently identified in differential gene expression analysis of tumor tissues when compared with the normal tissue, and have been attributed to differences in proliferative and metabolic rates (8, 32). The absence of such genes in our analysis probably reflects the use of HOSE and CSOC cells, which are morphologically indistinguishable and display similar growth characteristics. This finding further emphasizes the use of HOSE cells as well-matched controls in comparative gene analysis to identify aberrant gene expression in CSOC cells.

Several of the genes identified in this study are noteworthy. OSF-2 was originally reported as a transforming growth factor β-inducible protein secreted in the extracellular matrix of the periosteum (33). The potential significance of OSF-2 in ovarian cancer is illustrated by the high degree of OSF-2 overexpression observed in both ovarian tumors and cultured CSOC cells. OSF-2 overexpression in CSOC cells may be a consequence of inappropriate transforming growth factor β signaling that can be seen in some CSOC cells (19, 34). Although the function of OSF-2 is not known, OSF-2 or a related protein, βig-H3, is believed to function as a matrix protein that promotes cell attachment (33, 35). One possibility is that OSF-2 expression may facilitate i.p. spread of cancer cells, which leads to a significant morbidity and mortality in women with ovarian cancer. STC is expressed at high levels in organs derived from the müllerian duct (36), and, therefore, the loss of STC expression in CSOC cells may reflect cellular dedifferentiation. Cx43 and Cx40, which encode gap junction proteins, were cloned as HOSE-specific genes. Decreased gap junction communication and loss of Cx43 expression have been reported in ovarian cancer (37) and may be related to the loss of epithelial cell features in cancer cells, as well as decreased cellular communication that is seen in many types of cancers.

In conclusion, the availability of primary epithelial cultures from both normal and malignant ovaries has enabled the identification of 60 differentially expressed genes, using a combination of subtractive hybridization and cDNA array expression studies. Despite the lack of observable phenotypic or growth differences between HOSE and CSOC cells, reciprocal expression of OSF-2 with STC, KRT19, and Cx43 was seen, reflecting differences in these cells at the molecular level. A large number of genes identified in this study encode transmembrane or secreted proteins (see Tables 2 and 3) that may be present in serum and could be used as marker for ovarian cancer. In addition, the genes identified in this study may provide clues to the cellular changes responsible for metastatic progression of ovarian cancer.

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Gene-Expression Profiling in Normal and Malignant Ovarian Epithelia

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Running Title: Gene expression in ovarian epithelial cells

Abstract

The majority of ovarian cancers originate from a thin layer of epithelial cells covering the surface of the ovaries. To detect specific molecular changes associated with malignant component of ovarian tumors, we have characterized the variations in gene expression between the primary cultures of ovarian epithelial cells derived from the normal and cancerous ovarian tissue, using oligonucleotide microarrays containing 12,625 probe sets for known genes or EST's. When the gene expression patterns were analyzed by either the class distinction algorithm or hierarchical clustering algorithm, a clear distinction between normal and tumor-derived cells was observed among the 30 primary ovarian epithelial cultures. By comparing the gene expression patterns, we detected 111 genes that were upregulated >2.5-fold in tumor-derived cells and 62 genes that were expressed at higher levels in normal epithelial cells. In addition, there were distinct molecular portraits characteristic for normal and tumor-derived ovarian epithelial cells. Use of cultured epithelial cells represents a novel approach to study gene expression in a cancer cell specific manner.

Introduction

Ovarian cancer related mortality ranks first among all gynecological malignancies in the United States. Approximately 23,000 women will be diagnosed with ovarian cancer each year and less than half of them will be alive at 5 years (1). If diagnosed early, the disease is highly curable with complete surgical resection and adjuvant chemotherapy. However, because of absence of specific symptoms in early stages of the disease and lack of efficient screening, two third of patients are diagnosed with advanced disease. The only serologic marker in use for ovarian cancer is CA125, which is helpful for monitoring disease progression, but lacks the sensitivity or specificity to be useful in screening (2). Therefore, finding molecules specifically associated with ovarian carcinogenesis can help to identify additional screening markers and/or potential therapeutic targets.

Ovarian cancer originates in the epithelial lining of the ovaries. Different histological patterns can be identified, sometimes intermixed within the same tumor. The serous papillary subtype is the most common, while mucinous, endometrioid, and clear cell variants are less often encountered (3, 4). A related entity, primary peritoneal carcinomatosis arises from extraovarian sites in the peritoneum and has a clinical behavior that mirrors advanced stage ovarian cancer. It often has mullerian features, indicating a common embryological origin in the celomic epithelium that gives rise to the peritoneal lining as well as to the ovarian surface epithelium. It has been hypothesized that ovarian cancer and primary peritoneal carcinomatosis arise from the mullerian vestiges within the ovaries (rete ovaris) or outside the ovaries (endosalpingosis) (4).

The molecular changes associated with ovarian tumorigenesis are not completely understood (5). Mutations in the *BRCA 1* and *2* genes, alterations of *p53*, and amplification of *HER2/neu*, *AKT2*, and *myc* have been reported (6-13). In addition, numerous genes have been reported to be either up or down regulated in ovarian tumors (14-19). Gene-expression comparison of ovarian epithelial cancers to a normal epithelial-only ovarian counterpart has been difficult to perform, as the epithelial layer of the ovaries comprises less than 1% of the mass of the organ. This problem, further confounded by the lack of premalignant dysplastic changes in the ovarian epithelium and the heterogeneity of the disease, has made it difficult to establish distinct molecular patterns. To circumvent some of these problems and to focus on cancer cell specific molecular changes, primary cells cultured from normal and cancerous ovarian tissue have been used as an ovarian cancer model for gene-expression profiling (10, 18). This model permits a direct comparison between normal and malignant epithelial cells, avoiding signals from non-malignant cells (*e.g.,* stromal cells, endothelial cells, immune cells) mixed within tumors.

In this study, we used oligonucleotide microarrays to characterize gene expression in tumor-derived malignant ovarian epithelial cells (CSOC, Cedar Sinai Ovarian Cancer) and normal ovarian epithelial cells (HOSE, Human Ovarian Surface Epithelia). The distinction between malignant and normal epithelial cells was verified using "neighborhood analysis" and class distinction utilizing a modified version of the algorithm described by Golub *et al* (20). In addition, using clustering methods previously described (21), we were able to generate 2 sample groups of similar molecular pattern, which separated out the majority of CSOC samples from HOSE samples. Included in our list of ovarian cancer cell specific transcripts are those that are

known to be associated with neoplastic transformation as well as transcripts that previously have not been associated with ovarian cancer.

Materials and Methods

Primary ovarian epithelial cell cultures. Epithelial cells were scraped or microdissected with scissors from the ovarian surface and cultured as previously described (22, 23). The majority of cell cultures used in these experiments represent early passages (< 11 passages). Their epithelial nature was verified by immunohistochemical (IHC) analysis using antibodies against cytokeratin (AE1:AE3; Roche, Indianapolis, IN), vimentin (Roche, clone v9) and factor VIII (Factor VIIIc, Calbiochem, San Diego, CA). Both normal and malignant primary cells were grown in identical culture conditions using the same culture media. To minimize differences across experiments, all cells were harvested when they reached ~85% confluency.

Oligonucleotide arrays. Total RNA was extracted from each culture using RNA STAT-60 reagent (Tel-Test Inc, Friendswood, TX) and purified using the RNeasy Mini Kit (Qiagen Inc, Valencia, CA). Double stranded cDNA was synthesized using the HPLC purified T7-(dT)24 primer (Genset Corp, La Jolla, CA) and cDNA Superscript choice system (Gibco BRL, Carlsbad, CA). Biotinylated cRNA was obtained using the Bioarray High Yield RNA Transcript Labeling Kit (Enzo Biochem Inc, Farmingdale, NY) and purified with Qiagen RNeasy Mini Kit columns (Qiagen Inc, Valencia, CA). 15 μg of labeled cRNA was incubated in 40 mM Tris-acetate, pH 8.1, 0.1 M potassium acetate, 30 mM magnesium acetate at 94℃ for 35 min and hybridized to U95Av2 Genechips (Affymetrix, Santa Clara, CA). The images were scanned with a Hewlett Packard Gene-array Scanner (Affymetrix) and visually inspected for uniformity. The expression level for each probe set was calculated using the Affymetrix Microarray Suite (version 4.0.1, Affymetrix) (24). Briefly, the expression level is based on an average of the differences between

the perfect match-mismatch probe pairs for each gene. The values of the perfect match-mismatch pairs outside 3 standard deviation from the mean intensity of a specific gene or EST were discarded as outliers (*i.e.*, Affymetrix parameter STP set at 3). The intensities across different chips were normalized to a target intensity of 2500 using global normalization scaling. All values below the intensity of the background (300) across experiments were set equal to the background.

Data analysis. All genes were ranked by their probability of being significantly differentially expressed between the two sample types as determined using the Student t-test. The fold change in the mean of each gene between the normal and malignant samples was also calculated. Genes with a p-value less than 0.05 and a fold change of at least two were investigated further. The "neighborhood analysis" (20) was carried out using the same measure of differential expression as used in the Student t-test, $t = \frac{|\mu_1 - \mu_2|}{\sqrt{\frac{\sigma_1^2}{n_1 - 1} + \frac{\sigma_2^2}{n_2 - 1}}}$, where μ_i, σ_i and n_i are the mean, standard deviation and sample number, respectively, of sample group i. Random groupings (or permutations) of the samples were generated with one group containing the same number of samples as the original normal group, and the other containing the same number as the original malignant group. Random groupings were performed 10,000 times, and the mean and various significance levels for the number of genes with measures of differential expression (t) greater than a threshold level were determined. The hierarchical clustering analysis (21, 25) was carried out using ~1,500 genes with significant variation across samples (as defined by a standard deviation (σ) greater than 1000 and a coefficient of variation (σ^2/μ^2) greater than 0.09). We also required genes to have an Affymetrix 'present' call in at least half of the experiments (24).

RT-PCR validation. The Superscript II system (Gibco BRL) was used for reverse transcription, starting with 5 μg of total RNA and oligo-dT primer (GIBCO BRL) in a 20 μl reaction volume. 0.5 μl of the RT product was used for PCR amplification using primers specific for Wnt5a, TGM2, PAI2, OSF2, and G3PDH. Initial denaturing at 94□C for 90 sec was followed by 27-30 rounds of sequential thermal cycles: 30 sec denaturing at 94□C, 30 sec annealing at 60□C, 30 sec extension at 72□C, followed by a final extension at 72□C for 10 min. The primer sequences are: Wnt5a (forward (F): GCAACAAGGTAATTGCGTGCCATTCAG, reverse (R): CAGTGATACGCTGCAACACCTCTGTG), TGM2 (F: GCTGTGAGGAATGCTCTGCAG, R: GCAACTAGTAGGTGCTTCACAATGGTG), PAI 2 (F: GAACTCAGATCCATTCTGAGAAGC, R: GCAATTCTGAGGCACACAGCTCATC), OSF2 (F: GTGGTAGCACCTTCAAAGAAATC, R: CCTGAGAACGACCTTCCCTTAATCG), and GAPDH (F GATTCCACCCATGGCAAATTCC, R: CACGTTGGCAGTGGGGAC).

Results

Primary cell cultures of normal (HOSE) and malignant (CSOC) ovarian epithelia. Nine HOSE and 21 CSOC cultures were used for analysis. These primary cells display a limited life span of 5-9 passages for HOSE cells and a slightly extended 10-15 passages for CSOC cells (22). The characteristics of the CSOC cell cultures and the tumors they were derived from are detailed in Supplemental Tables 1 and 2. As previously described, these primary ovarian epithelial cells were fibroblast-like in appearance and displayed no specific morphological features or growth characteristics that would distinguish the tumor derived epithelial cells from the normal ovarian epithelial cells (data not shown; 22, 23).

Gene expression-based distinction of HOSE and CSOC cells. We compared gene expression differences between these two groups to differences between randomly generated groups of equivalent sizes to demonstrate that gene expression measurements can be used to distinguish normal from malignant ovarian epithelial cells. This approach, which has been referred to as "neighborhood analysis" (20), allows one to establish whether the correlation of observed gene expression in HOSE and CSOC samples to the normal/malignant classification is stronger than would be expected by chance. For each grouping, genes with expression difference of > 2-fold between the HOSE and CSOC samples were ranked by a measure of their p value from Student t test. Based on this analysis, the normal/malignant classification had more genes with significant expression differences than 0.1% of the random classifications (Fig 1). Thus, the differences between malignant and normal ovarian epithelial cells are clearly reflected in their overall gene expression patterns.

Genes differentially expressed in HOSE and CSOC cells. When the two groups (HOSE and CSOC) were compared, 2,176 genes had levels of difference in expression that reach statistical significance, based on the two-tailed Student t-test ($p < 0.05$). Within this group, there were 111 genes upregulated >2.5-fold in the cancer cells and 62 genes with >2.5-fold higher levels of expression in the normal cells (Supplemental Tables 3 and 4). The complete lists of genes that are differentially expressed are available at (http//www.cancer.mednet.ucla.edu/ovarian/).

Genes previously reported to be upregulated in ovarian cancer (e.g, *ALP*, *PAI-2*, *COL11A1*, *osteoblast specific factor-2 (OSF2)*, *cyclin B*) and novel genes not previously associated with ovarian tumors (*e.g., transglutaminase* (*TGM2*), *RAGE-3*, *PDGF receptor, ACLP*, *Dri42*, *TSG-14*, *Wnt5a, frizzled 7*, *biglycan*) were among the genes found to be overexpressed in CSOC cells. Genes strongly correlated with the HOSE phenotype were equally diverse. Genes such as *StAR*, *adipophilin, fibulin–2,* and *prostacyclin synthetase* encode proteins known to be expressed in ovarian tissue. Other HOSE-specific genes appear to reflect the epithelial nature of the HOSE cells (*e.g., PEM, plakophilin, merocin, Muc1*). Additionally, *BST2*, *FGF9*, and *Smad6* were also preferentially expressed in the normal cells.

Hierarchical clustering of CSOC and HOSE gene expression. To visualize the gene expression distinction between malignant and normal ovarian epithelial cells, we used hierarchical clustering and color-based representation of expression levels (Fig 2). Whereas the "neighborhood analysis" sorts gene expression levels by their degree of correlation to the normal/malignant classification, the hierarchical clustering algorithm groups experimental

samples according to the overall similarity in their gene expression pattern. The dendrogram pictured in Fig 2B illustrates the main segregation of the gene expression patterns. The majority (26 out of 30) samples segregated correctly to normal and malignant clusters, according to their original tissue diagnosis. Three of the CSOC samples (C889, C858 and C918) clustered on a sub branch next to the HOSE group, while the remaining CSOC cells clustered together. One of the HOSE samples (H263) clustered with the CSOC samples, on a branch situated in the vicinity of the HOSE cluster. This unbiased separation of the majority of CSOC and HOSE samples by hierarchical clustering suggests a clear distinction between the malignant and normal phenotype, which we attribute to the fundamental differences between the tissues of origin for these primary cells.

The main subgroups of genes driving the separation between malignant and normal ovarian epithelial cells, as determined by hierarchical clustering, are shown in Fig 2C-F. As expected, some of the genes recognized here included genes that were also selected, based on the *t*-test, as being preferentially expressed in HOSE or CSOC. For presentation purpose only a segment of each cluster is shown. One group of genes separating out the HOSE cluster from the rest of the samples is comprised of genes that are highly expressed in the normal cells relative to the malignant cells (*e.g., gap junction protein, carbonic anhydrase IX* and *XII, stanniocalcin, fibulin-2, plakophilin*) (Fig 2C). Also included in this cluster are 2 potential tumor suppressor genes, *Mxi1* and *DOC1*.

Within the cancerous sample division, there were additional subgroups specifically associated with high expression levels for certain genes. Statistically significant biological

distinctions between subgroups of cancerous samples could not be established, but we note several "molecular portraits". For instance, a subgroup of 7 samples (C843, C846, C839, C824, C823, C834, and C817) was characterized by intense expression of the genes encoding *proteasome-related proteins*, *nm23*, *placental protein 15*, and *karyopherin alpha 6* (Fig 2D). The second subgroup of 4 samples (C824, C823, C834 and C817) clustered together because of high expression levels of genes that are associated with cell proliferation (*e.g., CDC2, cyclinA2, cyclinB1, CDC28 protein kinase 2, CDC20*) (Fig 2E). This fraction of samples may represent an actively cycling group of tumor cells with more aggressive behavior. The third subgroup of 6 samples (C886, C844, C917, C866, C798 and C857) clustered together mainly due to overexpression of genes encoding proteins associated with the extracellular matrix and cell adhesion (*e.g., OSF2, byglycan, procollagen I N-proteinase, integrin β1 like protein, band 7.2 protein, lumican*) (Fig 2F).

RT-PCR validation of cancer-specific gene expression. For a few selected genes, we validated the level of expression from the array analysis through RT-PCR. We note a correlation between the amount of RT-PCR product and the level of expression as determined using microarrays (Fig 3). While the RT-PCR method does not provide quantitative correlation, especially for those with high expression levels, differential expression between CSOC and HOSE cells can still be appreciated for each of the tested genes (*Wnt5a*, *OSF2*, *PAI2* and *TGM2*) (Fig 3). The level of expression of *OSF2, TGM2,* and *Wnt5a* was also verified for RNA obtained from ovarian tumor samples, indicating that the expression pattern of at least some genes is maintained during *in vitro* expansion of epithelial cells from the tumor (data not shown).

Discussion

Defining molecular patterns associated with specific tumors is becoming an increasingly common process (26-29). The prevailing practice in the field is to use clinically obtained tumor samples as the starting material and carry out gene-expression profiling to define molecular signatures that will classify tumor subtypes. While such approaches have proven to be useful in the study of hematological malignancies (26) or childhood tumors (29), where tumor samples tend to be relatively homogeneous, the molecular classification of adult epithelial tumors has been challenging due to heterogeneity of the tumor tissue. In a comparative analysis of normal and tumor samples, this problem is further confounded by the difficulty in obtaining the normal epithelial cells, free of stromal components.

To minimize the problems related to heterogeneity in the cellular components of tumor or normal tissue, we utilized a human ovarian cancer model that is based on *in vitro* expansion of malignant and normal ovarian epithelial cells. In this model, all cultured cells are epithelial, based on IHC staining, and grossly uniform, as reflected in their phenotype and growth characteristics. Reliance on cultured cells introduces problems with *in vitro* selection bias and variability in culture conditions. This *in vitro* selection bias may have led to some of the gene expression variation seen in different HOSE and CSOC samples and incorrect clustering of 3 CSOC and 1 HOSE sample (see Fig 2). However, these problems are offset by the fact that the use of expanded primary cultures is more likely to reveal gene expression changes that are cancer cell specific.

In our study, two lines of evidence indicate that phenotypically distinct cells are cultured from the tumor than the normal ovary in spite of their apparent similarities in cell morphology and growth characteristics. First, when the gene expression levels were sorted by degree of their correlation to the class distinction (*i.e.,* CSOC *vs.* HOSE) by the "neighborhood analysis" method, there was an unusually high density of genes that followed the idealized pattern according to this class distinction, as compared to equivalent random patterns. Second, the hierarchical clustering algorithm uncovered a clear separation between the normal cells from malignant cells. This separation represents the main division of our clustering tree, followed by smaller subdivisions distinguishing separate fractions within the CSOC sample group. These two statistical findings, together with the previous findings that CSOC cells, but not HOSE cells, contain chromosome abnormalities (18), indicate that our ovarian cancer model based on cultured epithelial cells is a valid system to study gene expression in ovarian cancer.

There were 111 genes (0.9% of 12,000) with mean expression values of > 2.5-fold higher in favor of malignant epithelial cells. A similar number of genes (62, 0.5% of 12,000) were expressed at higher levels (> 2.5-fold) in the normal epithelial cells than the cancer cells. The list of differentially expressed genes includes genes that have previously been associated with the neoplastic process as well as those that have not been recognized as transformation related genes in ovarian or other human cancer. Among these, *OSF2*, which encodes a secreted protein related to the axon guidance molecule fasciclin-1 of insects (13, 30), ranks high (p <0.0005). This putative adhesion molecule, which may facilitate tumor spread along the peritoneal surface, was previously reported to be an ovarian cancer specific gene on the basis of experiments utilizing cDNA-based representational difference analysis (18). The *Wnt* genes encode a widely

distributed family of secreted growth factors critical for embryonic pattern formation and cell lineage differentiation (31). While Wnt5a protein is not known to be transforming, it is implicated in the regulation of progenitor cell proliferation (32) and upregulated in breast cancer (33). The frizzled family of transmembrane proteins functions as receptors for the Wnt proteins (31). In our analysis, Wnt5a and frizzled 7, a potential ligand-receptor pair, were found to be overexpressed in CSOC cells relative to HOSE cells, which suggests that activation of the Wnt pathway may play a role in cell proliferation and malignant transformation in ovarian cancer. TGM2 is a GTP-binding protein that participates in protein transamidation (34). Our finding that TGM2 is overexpressed in malignant cells, together with recent reports on its association with Rho signaling and cell cycle (35), and apoptosis (36), suggests that TGM2 may play a role in cancer cell proliferation.

Analysis of genes with stronger expression in HOSE cells offers insight into both genes that may play a permissive role in the neoplastic process and those that may govern cellular differentiation. The only gene with tumor suppressor function that is revealed in our study is Mxi1, which has been shown to inhibit cell proliferation and to induce of cell cycle arrest in prostate tumor cells (37, 38). FGF9 and Smad6, a protein implicated in TGF beta signaling and potentially a FGF synergistic molecule (39, 40), have 2 fold higher intensities in HOSE, suggesting that they may play a role in the proliferation of epithelial ovarian cells and that cancer cells may have circumvented this requirement. Other genes with strong signal in HOSE cells include *plakophilin*, *stanniocalcin* , *adipophilin* , *BST2*, *StAR*, *PACE4, prostacyclin synthetase,* and *Muc1.* These genes have previously been shown to be expressed in a tissue-restricted manner and may represent differentiation specific genes (41-45). The down regulation of these

genes, therefore, may reflect cellular de-differentiation associated with the neoplastic transformation.

We believe our model using normal and malignant epithelial cells expanded in culture represents a novel approach to identify genes that are differentially expressed in a cancer cell specific manner and will complement other gene expression studies that utilize clinical tumor samples. Genes that are differentially expressed in a cancer cell specific manner will provide insights to the initiation and progression of ovarian carcinoma. In addition, these genes represent potential molecular targets for the development of diagnostic or therapeutic tools for ovarian cancer.

Acknowledgement

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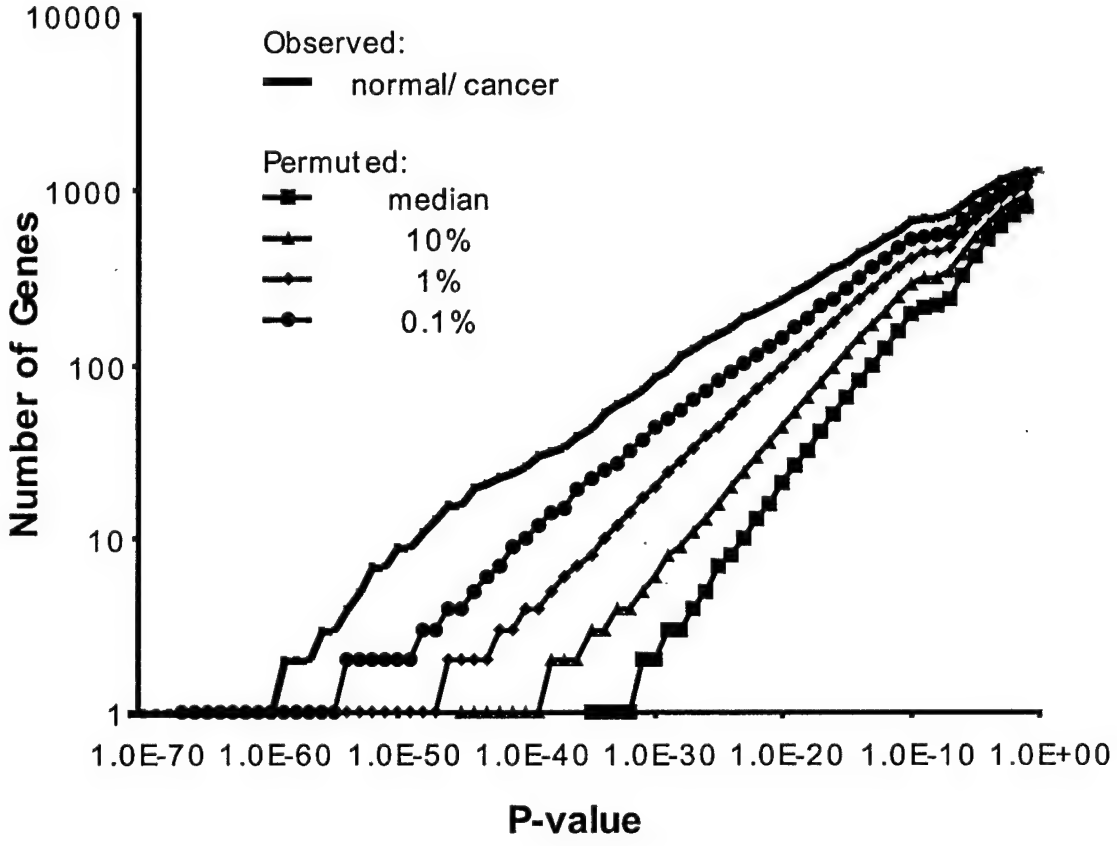
Figure legends

Figure 1. The gene expression differences between malignant and normal ovarian epithelial primary cultures are statistically significant. The number of genes with a measure of differential expression, *t* (Materials & Methods), greater than various threshold levels were calculated for the normal/malignant classification and are plotted (solid line). Random groupings were also generated and tested. The median and various confidence levels for these random groupings are also plotted. A confidence level of 1%, for example, indicates that 1% of the random groupings had a higher number of genes that were differentially expressed at the indicated *p* value.

Figure 2. (A) Differential expression profile in 30 samples of cultured CSOC and HOSE cells. Columns represent individual samples and rows represent genes. Each cell corresponds to the level of expression of a particular gene in a given sample. All values have been normalized to a mean of 0 and a standard deviation of 1. A visual dual color code is utilized with red and green indicating relatively high and low expression levels, respectively. Cluster and Treeview programs were used to generate these figures. The scale of color saturation, which reflects the gene expression levels, is shown below. (B) Dendrogram illustrating the separation of samples based on their degree of similarity as measured by the Pearson correlation coefficient. (C) Group of genes with high level of expression in HOSE cells. (D-F) Groups of genes with relative high expression in CSOC cells. Each group is empirically denoted as the "proteolysis" group (D), "proliferation" group (E), and "ECM/cell adhesion group" (G), based on the predominance of particular type of genes in each group. Commonly used alias of the gene name is provided with the corresponding unigene number. The scale at the bottom shows the relationships between

color saturation and gene expression levels.

Figure 3. Validation of gene expression differences between CSOC and HOSE cells. (A) Expression differences detected by microarrays were verified by RT-PCR for Wnt5a, PAI2, TGM2, and OSF2. GAPDH was used as a control to standardize RT-PCR. H246, H254 and H274 are normal ovarian epithelial cells (HOSE). C874, C886, C889, C839, C843, C846 and C881 are malignant ovarian epithelial cells (CSOC). Sk-Ov-3 is an ovarian carcinoma cell line. (B) Gene expression levels as determined by microarrays are shown.


10000
1000
100
10
1
Number of Genes
Observed:
normal/ cancer
Permuted:
median
10%
1%
0.1%
1.0E-70 1.0E-60 1.0E-50 1.0E-40 1.0E-30 1.0E-20 1.0E-10 1.0E+00
P-value

A

B

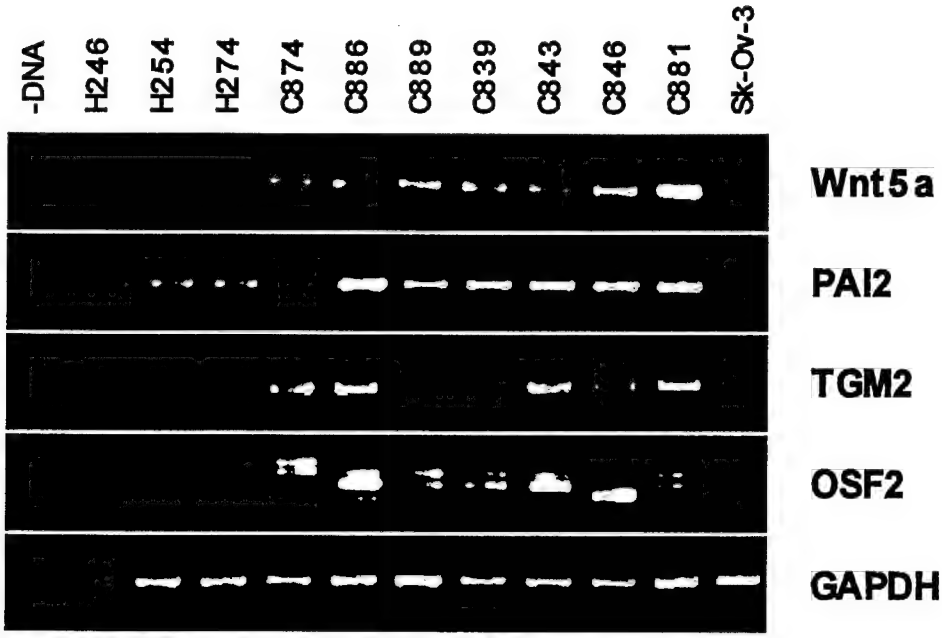
Solute carrier family 23, mbr 1 (Hs.82042)
ATP binding cassette, subfamily C, mbr 3 (Hs.90786)
Serine protease 11, IGF binding (Hs.75111)
Serine protease 11, IGF binding (Hs.75111)
Calcium channel, voltage dependent alpha 1H (Hs.122359)
KIAA0367 (Hs.23311)
Gap junction protein alpha 1 (Hs.74471)
Platelet phosphofructokinase (Hs.99910)
Solute carrier family 6, mbr 8 (Hs.187958)
Peroxiredoxin 2 (Hs.146354)
Forhead box 03A (Hs.14845)
Acyl Coenzyme A dehydrogenase, very long chain (Hs.82208)
RNA binding protein 2 (Hs.211610)
Eukaryotic translation elongation factor 1 alpha 2 (Hs.2642)
BCL2 adenovirus E1B interactin protein like (Hs. 132955)
Jagged 2 (Hs.166154)
Cyclin D2 (Hs.75586)
DKFZP586H2123 (Hs.55044)
Carbonic anhydrase XII (Hs.5338)
Adaptor related protein complex 1, gamma 1 subunit (Hs.5344)
Carbonic anhydrase IX (Hs.63287)
Adipose differentiation related protein (Hs.3416)

Testis enhanced gene transcript (Hs.74637)
Retinoblastoma binding protein 4 (Hs.16003)
Karyopherin alpha 6 (Hs.169475)
Placental protein 15 (Hs.151734)
Tyr/Trp monooxygenase activation protein (Hs.75103)
Phosphotidylinositol transfer protein, beta (Hs.7370)
PAC845024, similar to PRAME(Hs.104991)
GDP dissociation inhibitor 2 (Hs.56845)
Synovial sarcoma, X breakpoint 1 (Hs.194759)
ARP 2/3, subunit 4 (Hs.149570)
NM23A (Hs.118638)
NM23A (Hs.118638)
NM23A (Hs.118638)
Proteasome 26S, ATPase 3 (Hs.250758)
Proteasome subunit beta type3 (Hs.82793)
Proteasome 26S non ATPase 13 (Hs.279554)
Proteasome 26S non ATPase 4 (Hs.148495)

CDC 2 (Hs.184572)
Karyopherin alpha 2 (Hs.159557)
CDC28 protein kinase 2 (Hs.83758)
CDC20 (Hs.82906)
Thyroid hormone receptor interactor 13 (Hs.6566)
Hypothetical protein MGC1780 (Hs.77550)
Cyclin A2 (Hs.85137)
Cyclin A2 (Hs.85137)
Minichromosome maintenance deficient 2 (Hs.57101)
Kinesin like-1 (Hs.8878)
Ser Thr kinase 12 (Hs.180655)
Pituitary tumor transforming 1 (Hs.252587)
Centromere protein F (Hs.77204)
CDK inhibitor 3 (Hs.84113)
Ubiquitin carrier protein E2-C (Hs.93002)
ZW10 interactor (Hs.42650)
Chromosome 20 ORF1 (Hs.9329)
Cyclin B1 (Hs.23960)

Osteoblast specific factor 2 (Hs.136348)
Deleted in liver cancer 1 (Hs.8700)
JM4 protein (Hs.29595)
Yip1p interacting factor (Hs.5809)
Signal recognition particle 19KD (Hs.2943)
Pyruvoyltetrahydropterin synthase (Hs.366)
Lipocortin pseudogene 2 (Hs.234757)
Endoplasmic reticulum protein retention, rec 3 (Hs.250696)
Biglycan (Hs.284264)
Flap structure specific enndonuclease 1 (Hs.4756)
Polycystic kidney disease 2 (Hs.82001)
Fer1L3-like 3 (Hs.234680)
KIAA0626 (Hs.178121)
Frizzled 7 (Hs.173859)
KIAA0938 (Hs.174188)
Procollagen I N-Proteinase (Hs.120330)
Integrin beta like 1 with EGF like domain (Hs.82582)
Purine-rich element binding protein A (Hs.29117)
KIAA0337 (Hs.45180)
Erythrocyte protein band 7.2 (Hs.160483)
KIAA0620 (Hs.105958)
Growth arrest and DNA damage inducible, beta (Hs.110571)
Pleomorphic adenoma 1 like (Hs.75825)
Solute carrier family 35, mbr 3 (Hs.159322)
Lumican (Hs.79914)

A



B

Gene	H246	H254	H274	C874	C886	C889	C839	C843	C846	C881
Wnt5a	1039	1909	103	1061	2136	1444	16117	28422	5052	20603
PAI2	77	5414	3670	77	108544	5847	6556	23078	27908	30547
TGM2	1858	-406	259	11868	27364	494	10144	89488	52446	34470
OSF2	470	1156	1466	16420	46724	2494	48591	41538	46088	34112

Immunoglobulins in Malignant Ascites Inhibit Adenoviral Infection of Tumor Cells: Implications for Adenoviral Gene Therapy

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Key Words: adenovirus, gene transfer, malignant effusions, immunoglobulin, ovarian cancer

Running Title: Inhibition of Gene Transfer by Malignant Effusions

Introduction

Both adenoviruses and retroviruses have been proposed as vectors for the therapeutic transfer of human genes *in vivo* as a treatment for human malignancies, as well as other human genetic diseases such as cystic fibrosis (Nielsen and Maneval, 1998). The use of adenoviral vectors has several advantages over retroviral vectors in terms of clinical application for cancer treatment, including: high titer production, high transduction efficiency, lack of requirement for cell replication for transgene expression, lack of insertional mutagenesis, and wide tissue tropism of viral infection (). Moreover, recent evidence suggests that there are inhibitors of retroviral infection in human body fluids which may further limit the utlility of retroviruses as a vehicle for therapuetic gene transfer. Biochemical separation of cell-free, malignant pleural fluid samples identified chondroitin sulfates as the major inhibitor of retroviral infection (Batra, et al.). Such inhibitors of viral infection *in vivo* have not yet been described for the adenoviruses. However, neutralizing anti-adenovirus antibodies have been shown to inhibit adenovirus transduction in animal models (). In addition, human sera containing anti-adenovirus antibodies has been shown to reduce infection of rat hepatocytes following adenovirus inoculation via the portal vein ().

Numerous clinical trials have been initiated for a variety of malignancies using adenoviral vectors containing human, wild-type p53 tumor suppressor gene cDNA (Clayman; Nielsen, Pegram, et al., 1999; Roth; Schuler et al., 1998). The results to date indicate that while p53 transgene expression is possible *in vivo*, delivery of adenoviral vectors is limited by the lack of penetration and distribution of adenovirus particles within solid tumor masses (Grace et al., 1999), and by the fact that administration of a very large number of viral particles requires that a sizeable fluid volume be administered into the tumor which is technically not feasible for many common cancers (i.e. lung cancer and brain tumors). Therefore, more recently attention has focused on the use of adenoviral vectors for malignancies which remain localized to a single anatomical site for long periods, and for cancers for which local-regional gene delivery mechanisms are

technically feasible and are more likely to be of benefit. For example, cancers of the head and neck are not often associated with distant metastases until late in their course, and cancers of the ovary, bladder, and pleura remain confined to a body cavity into which adenovirus suspensions may be instilled directly. Of these potential clinical targets, ovarian cancer is one of the most attractive because of the high prevelence of p53 mutations in this disease, the non-hematogenous pattern of spread of epithelial ovarian cancer - which remains confined to the peritoneal cavity throughout much of its clinical course, and the lack of efficacy of existing treatment for the disease (Nielsen, Pegram, et al., 1999). In preclinical models, replication-deficient, recombinant adenovirus containing the human p53 tumor suppressor gene cDNA (rAd-P53) (Wills et al., 1994) has therapeutic efficacy against a wide range of human tumor types containing nonfunctional p53, and it has enhanced activity in combination with many chemotherapeutic drugs (Anderson et al., 1998; Gurnani et al., 1999; Nielsen, and Maneval, 1998; Nielsen et al., 1998a; Nielsen et al., 1998b; Nielsen et al., 1999). rAd-P53 is currently undergoing phase II/III clinical trials in ovarian cancer.

A majority of patients with ovarian cancer will develop malignant peritoneal effusions (ascites) at some time during the course of their disease. The presence of ascites is correlated with tumor burden and is an adverse prognositc factor in patients with ovarian cancer (). In this work we sought to determine whether there are inhibitors of adenovirus transduction in malignant ascites fluid from ovarian cancer patients as there are for retroviruses in patients with malignant pleural effusions (Batra, et al.).

Materials and Methods

Adenoviruses

Construction and propagation of *E. coli* β-galactosidase adenovirus (rAd-β-gal) have been described previously (Wills et al., 1994). The concentration of total viral particles (PN) was determined by measuring absorption at 260 nm (Huyghe et al., 1995). The concentration of total PN in control vector batches was determined by Resource Q Anion Exchange HPLC assay (Shabram et al., 1997). Infectious PN was determined by measuring the concentration of viral hexon protein positive 293 cells after a 48 hr. infection period (Huyghe et al., 1995).

Human tumor cell lines

Primary ovarian tumor cells (Cedars Sinai Ovarian Cancer Cells (CSOC)) were established from ascites and solid tumor cultures and characterized as previously described ().These cells were maintained in McCoys media with 10% fetal calf serum and 1% penicillin/streptomycin at 37°C and 5% CO_2. All other cell lines were obtained from the American Type Tissue Culture Collection (ATCC) (Rockville, MD). MDA-MB-231 human mammary tumor cells, which carries an Arg-to-Lys mutation in codon 280 of the p53 gene ($p53^{mut}$), were cultured in DMEM (GIBCO/Life Technologies, Grand Island, NY) with 10% fetal calf serum (FCS; Hyclone, Logan, Utah) at 37°C and 5% CO_2. SK-OV-3 human ovarian tumor cells ($p53^{null}$) were cultured in Eagle's MEM plus 10% FCS at 37°C and 5% CO_2.

Collection of human malignant effusions/ascites

All clinical speicmens were obtained under an IRB-approved clinical protocol and following patient informed consent. Malignant effusions were obtained under sterile conditions in the operating room at time of initial cytoreductive surgery. Fluid specimens were centrifuged for 20 minutes at 2000 rpm, and the resultant supernatants were collected, aliquoted and stored at -20°C.

Measurement of Adenoviral Transduction Efficiencies in Human Malignant Ascites

Susceptibility of target cells to adenoviral infection was determined using a replication deficient, recombinant adenoviral vector containing an *E. coli* β-galactosidase reporter construct driven by a human cytomegalovirus (CMV) promoter (rAd-β-gal). MDA-MB-231 or CSOC cells were plated in 24 well plates at 5×10^4 cells/well in 1 ml of McCoys media, and were allowed to adhere overnight at 37°C. The cells were washed

with phosphate buffer saline and then allowed to incubate with rAd-β-gal (1×10^9 particles/ml) for 24 hours at 37°C and 5% CO_2 with defined concentrations of human malignant ascites fluid or control media. Cells were washed, fixed with 3.7% formaldehyde solution, and stained with an X-gal solution (Boehringer Mannheim Corp, Indianapolis, Indiana). After 24 hours, the cells were washed and counted by light microscopy. Quantitation was performed in triplicate by two observers.

Transgenic MidT tumor cell derivation and in vivo mouse study

Mammary tumors from female MMTV-Polyomavirus Middle T (PyV MidT) transgenic mice (Guy, Cardiff, and Muller, 1992) were passaged through FVB immunocompetent, syngeneic host mice. Cells from MidT/FVB tumors were dispersed, then cultured in 90% Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY) with 10% fetal bovine serum (FBS; GIBCO) (Nielsen et al., 2000). The mixed cell populations were subsequently cloned and the MidT2-1 cell line selected for use based on the criteria outlined in Nielsen et al. (2000). Culture media from MidT2-1 cells were collected in sterile test tubes, centrifuged at 1000 x g for 15 minutes, and the supernatents frozen at –80 °C.

Immunocompetent FVB mice were from the SPRI colony. Immunodeficient C.B.17/ICR-SCID mice were purchased from Taconic Farms (Germantown, NY). All mice were maintained in a VAF-barrier facility. All animal procedures were performed in accordance with the rules set forth in the N.I.H. Guide for the Care and Use of Laboratory Animals and were approved by the SPRI Animal Care and Use Committee. Some FVB mice were preimmunized with 1×10^{10} PN adenovirus at least one week before the injection of tumor cells in order to stimulate the production of anti-adenovirus antibodies. Female SCID and FVB mice were injected with SK-OV-3 or MidT2-1 cells intraperitoneally. After tumors were established, some mice were injected with five daily doses of intraperitoneal rAd-β-gal (1×10^{10} PN). Tumorigenesis was allowed to progress to an advanced stage when ascites formation occurred, then mice were sacrificed and

ascites collected in sterile test tubes. Samples were centrifuged at 3000 x g for 15 minutes and the supernatents frozen at –80 °C.

AntiAdenoviral Antibody Detection

Ninety-six well plates were coated with 5.0×10^9 particles/ml ZZCC recombinant adenovirus for 12 hours at room temperature. The plates were washed and blocked with buffer containing 1% BSA and 0.05% Tween 20. Plates were then washed and incubated with a serially diluted ascites fluid beginning at a 1:200 dilution for 1.5 hours at room temperature. Each ascites specimen was run at the following dilutions: 1:200, 1:400, 1:800, 1:1600, 1:3200, Plates were washed and incubated with Peroxidase conjugated Goat-ant human IgG for 1 hour at room temperature. Plates were washed, then incubated for 30 minutes with ABTS peroxidase substrate (Boehringer Mannheim) and read at OD 450 nm.

Anti-adenovirus neutralizing antibody assay

Mouse ascites was collected at the time of necropsy, heat-inactivated at 56 C for 40 min., and serially diluted with MEM in two-fold steps starting at 1:20. Serum dilutions (100 µl) were mixed 1:1 with a recombinant adenovirus expressing ß-galactosidase (8.0 x 10^8 PN/ml), incubated for 1 hour at 37 C, and applied to a subconfluent monolayer of HeLa cells (ATCC). After 90 minutes at 37 C, 50 µl of MEM containing 10% FBS was added to each well and the cells were incubated overnight at 37 C. Cells were fixed in 0.2% glutaraldehyde and assayed for ß-galactosidase expression the following day. Control wells containing no ascites stained 100% for ß-galactosidase expression. A positive control sample, known to have neutralizing capacity, was run in each assay. The mean titer of neutralizing antibody from duplicate wells was reported as the highest dilution at which 50% or less of the cells stained blue. The serial dilutions tested were 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, and 1:2560.

Purification of antiadenovirus antibody from human malignant ascites

Anti-adenovirus antibody was purified from human malignant ascites by protein A/G

column chromatography as described (). The inhibitor of rAd-Bgal transduction was isolated by DEAE-C ion exchange column chromatography. The inhibitory fraction was then subject to SDS-PAGE. Western blot analysis was performed as described () using human anti-IgG K as the detection antibody ().

Results

Human malignant ascites inhibits in vitro transduction by adenovirus containing the B-galactosidase reporter gene in a dose and schedule-dependent fashion.

In order to ascertain whether malignant ascites inhibits adenovirus infection, the cell-free fraction of malignant ascites collected from eighteen patients with papillary serous ovarian carcinoma and from patients with other malignancies were allowed to incubate, in the presence of adenovirus, with MDA-MB-231 breast carcinoma cells which are efficiently infected by adenoviral vector containing the B-galactosidase reporter gene. Admixture of 50% ascites with cell culture media completely blocked adenovirus infection of MDA-MB-231 cells at a viral concentration of 1×10^9 particles/ml, and a 20% admixutre of ascites with media inhibited adenovirus transduction by >50% in 15 of 18 samples analyzed (Figure 1A). Inhibition of adenovirus transduction was not a result of decreased pH in ascites samples since 50/50 admixture with culture media resulted in physiologic pH in all of the samples analyzed (data not shown). To determine whether the inhibitory effect of malignant ascites was limited to ascites samples from ovarian tumors of papillary serous histology, effusions from other neoplasms were analyzed including: 1) appendiceal carcinoma, 2) gastric carcinoma, 3) endometrial adenocarcinoma, 4) primary peritoneal carcinoma, 5) ovarian mixed mesodermal tumor, 6) ovarian clear cell carcinoma, 7) ovarian mucinous carcinoma, and 8) pleural effusion from an ovarian papillary serous adenocarcinoma. A 50% or greater inhibition of adenoviral gene transfer occurred at a 50% dilution in all the specimens analyzed from the various malignancies indicating that inhibition of adenovirus transduction is not restrcicted to ovarian cancer ascites (Figure 1B).

The inhibitory effect of ovarian cancer ascites was dose-dependent, and the demonstration that there is no inhibitory activity contained in cultured media from primary human ovarian carcinoma cells suggests that the inhibitory factor is host-derived

as opposed to being derived from the tumor cells themselves (Figure 2A). Heat inactivation of complement by allowing ascites samples to incubate at 65°C for one hour did not result in attenuation of inhibitory activity of adenovirus transduction of MDA-MB-231 cells (data not shown). In scheduling experiments, admixture of ascites with adenovirus simultaneously, or for one hour prior to exposure to target MDA-MB-231 cells, completely blocked adenovirus transduction; whereas preincubation of ascites with MDA-MB-231 cells, followed by a wash step and then exposure to adenovirus, resulted in no inhibition of adenoviral transgene expression (Figure 2B). These results indicate that the inhibitory factor is most likely directed against adenovirus and not directed against the target cell line since preincubation of target cells with ascites did not result in inhibition of adenovirus transduction. To assure that the inhibitory effect of ascites on adenoviral infection was not restricted to transduction of MDA-MB-231 cells, we analyzed the transduction efficiency of four primary ovarian carcinoma cell cultures in the presence of matched ascites samples taken from the same patient from which the corresponding ovarian tumor cell cultures were derived. In these cases, adenoviral gene transfer was inhibited in the primary tumor cell cultures by the presence of 50% ascites identically as with MDA-MB-231 cells as the target for adenoviral transfection (Figure 3). Therefore inhibition of adenovirus transduction was not specific for the cell type serving as the target for gene transfer.

Inhibition of adenovirus transduction in murine malignant ascites correlates with anti-adenovirus humoral immune response in immunocompetent mice.

Since IgG is a known component of malignant ascites and because neutralizing anti-adenovirus antibodies have been described previously in serum samples, we sought to determine whether the inhibitory factor(s) in ascites fluid could be a result of humoral immunity against adenovirus. For these experiments we chose an immunocompetent mouse model of peritoneal carcinoma (Nielsen et al., 2000). In this model, MidT2-1 peritoneal tumors are distributed throughout the peritoneal cavity as both small and large nodules. They were commonly found on the peritoneal surface of abdominal organs, but are also found invading the surfaces of the diaphragm, liver, pancreas, and intestines. All mice develop bloody ascites as a late-stage manifestation of their disease. For these

experiments, ascites samples collected from adenovirus-naive mice were compared directly to ascites samples collected from mice that were immunized with adenovirus. As shown in table 1, 20% ascites from immunodeficient SCID mice bearing human SK-OV-3 ovarian tumor xenografts had no effect on the transduction of MDA-MB-231 human breast tumor cells by recombinant adenovirus *in vitro*. By contrast, there was a 38% reduction in adenovirus transduction efficiency when MDA-MB-231 cells were cultured with 20% ascites from either SCID or immunocompetent FVB mice bearing syngeneic MidT2-1 mouse mammary tumors. This reduction was observed despite the fact that these mice were never exposed to adenoviruses, but was not observed when culture medium from MidT2-1 cells was substituted for mouse tumor ascites (data not shown). This suggests the production of a low efficiency inhibitory factor(s) by MidT2-1 tumors *in vivo*. However, the predominant inhibitory effect (100% inhibition) was only observed in tumor ascites from immunocompetent mice exposed to multiple doses of recombinant adenovirus. These mice also developed high levels of neutralizing antibodies against adenovirus (Table 1). These results demonstrate that ascites derived from adenovirus-naive mice had little inhibitory activity against adenovirus transduction, whereas ascites samples from tumor-bearing mice immunized against adenovirus displayed a marked increase in inhibitory activity of the same magnitude seen in the experiments conducted with human ascites specimens (Table 1). Furthermore, the demonstration that the inhibitory activity of murine ascites correlates with the presence of anti-adenovirus antibodies measured by ELISA strongly supports that the hypothesis that the inhibitor is a humoral immune factor. (Table 1).

Demonstration of anti-adenovirus antibodies in human ascites by ELISA.

To determine whether human malignant ovarian cancer ascites contains anti-adenovirus antibodies, we measured anti-adenovirus antibody titers by ELISA. Eighteen of eighteen samples analyzed contained measurable amounts of anti-adenovirus antibody indicating prior exposure to type 5 adenovirus in all of the patients in whom ascites was collected (Table 2). This finding is consistent with the high prevelence of anti-type 5 adenovirus antibodies in serum of adults in the general population (). We were not able to demonstrate correlation between antibody titer and inhibition of adenovirus transduction,

perhaps secondary to the relatively small sample size, the fact that some ascites samples with low titer inhibited adenovirus transduction as efficiently as samples with high titers, and some of the samples with high titer antibodies may contain a significant fraction of non-neutralizing antibodies which may not inhibit viral infection. The presence of anti-adenovirus antibodies in all of the samples tested is consistent with the hypothesis that inhibition of adenoviral transduction by ascites is mediated by antibodies.

Removal of inhibitor of adenovirus transduction from malignant ascites by protein A/G.

Ascites samples with inhibitory activity against adenovirus transduction were allowed to pass through a column containing protein A/G. Ascites eluate from the column had marked attenuation of adenovirus inhibition, and purification and analysis of the the protein A/G column-bound fraction demonstrated that inhibitory activity could be reconstituted from this material (Figure 4). Furthermore, precipitation of immunoglobulin from ascites with 60% ammonium sulfate completely abbrogated inhibitory activity (data not shown). Taken together these data strongly suggest that the inhibitor of adenovirus transduction in human ascites is an immunoglobulin.

Direct Purification and Characterization of Adenovirus Transduction Inhibitor from Human Ascites.

The major inhibitory component of adenovirus transduction in malignant acites was purified by DEAE-C ion exchange chromatography (Figure 5A). The inhibitory activity was largely confined to fractions 6-11 following ion-exchange separation. Analysis of this fraction by SDS gel electropheresis revealed a protein band with molecular weight (~150Kd) which migrates at the same position as control human IgG. This band was not visible in fractions of longer retention times. Under reducing conditions, two bands were present corresponding to the molecular weights of light and heavy chains of control IgG protein (Figure 5B). We then directly confirmed that these bands are light and heavy chains of human IgG by Western blot analysis of the reduced proteins (Figure 5C). These data demonstrate that the major inhibitory component of adenovirus transduction in human malignant ascites derived from ovarian carcinoma is human anti-adenovirus IgG.

Discussion

There are numerous potential physiological barriers which may mitigate the efficiency with which adenoviruses infect tumor cells *in vivo*. These include anatomical barriers such as the location of tumor deposits within visceral organs which are difficult to access for direct injection of viral vectors, large tumor size resulting in lack of penetration of large viral particles into solid tumor masses (Grace,et al, 1999 Am J Pathol), and the capillary endothelial barrier which might prevent diffusion of vector into tumors when vector is administered via the hematogenous route. Furthermore, once adenoviral vectors have reached the tumor target, efficient infection may be limited by lack of expression of adenovirus receptors in tumor cells (). Adenovirus infection and therapeutic gene transfer into tumor targets may also be limited by both the humoral and cellular immune responses against the adenovirus (). In this work we sought to determine whether there are inhibitors of adenoviral infection in human malignant ovarian cancer ascites fluid, analogous to those which have been described for retroviruses in malignant pleural effusions in lung cancers (Batra, et al.). We were able to demonstrate potent inhibitors of adenovirus infection of MDA-MB-231 breast cancer cells *in vitro* with solutions containing 50% ascites in cell culture media. The inhibitory activity was not restricted to ovarian cancer ascites fluid as malignant ascites from a variety of human malignancies also inhibited adenoviral infection. Conditioned media from ovarian carcinoma cell lines did not inhibit adenoviral infection *in vitro* suggesting that the inhibitor was host derived rather than being derived from tumor cells (Figure 2A). Using an immunocompetent mouse model of malignant ascites, we have demonstrated that inhibitory activity against adenovirus could be induced by prior inoculation of mice by type 5 adenovirus, and that the inhibition corresponded to induction of anti-adenovirus antibodies in mouse ascites fluid (Table 1). Moreover, we detected anti-type 5 adenovirus antibodies by ELISA in all human ascites samples tested. Finally, direct purification of the major inhibitory fraction of adenovirus infection by ion exchange chromatography yielded human IgGs. We have identified other minor inhibitory fractions of adenovirus infection by column chromatography suggesting that there may be other non-IgG

inhibitors in ascites fluid as well (Figure 5A), but these constitute only a small fraction of the total inhibitory activity found in ascites. Further study of these components is warranted to identify other potential factors which may limit adenoviral infection of human tumor targets *in vivo*.

These results demonstrate that the major inhibitor of adenovirus infection in human malignant ascites is preexisting anti-adenovirus antibodies caused by prior type 5 adenoviral infection. Our results suggest that to improve adenovirus-mediated gene delivery to ovarian tumor cells *in vivo*, removal of ascites fluid prior to intraperitoneal infusion of adenoviral vector would be desirable. Preliminary studies from Phase I clinical trials of adenovirus-mediated gene transfer to ovarian tumors *in vivo* has demonstrated that transgene expression is possible despite the existence of anti-adenovirus antibodies, as long as the dose of adenovirus is very high ($>\sim 10^{11}$ viral particles/dose) (Buller, et al.). This apparent threshold dose has been seen with other tumor types, and may represent a minimal vector dose required to overcome the effects of neutralizing anti-adenovirus antibodies (). Based on our experimental results, future efforts to improve efficiency of adenovirus-mediated gene transfer should focus on means of avoiding the humoral anti-adenovirus immune response such as the development of less immunogenic vectors, liposomal encapsulation of adenovirus particles, and possibly the use of immunosuppressive drugs to blunt the anti-adenovirus immune response ().

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Figure 1A. Inhibition of Ad Transduction by Malignant Ovarian Cancer Ascites

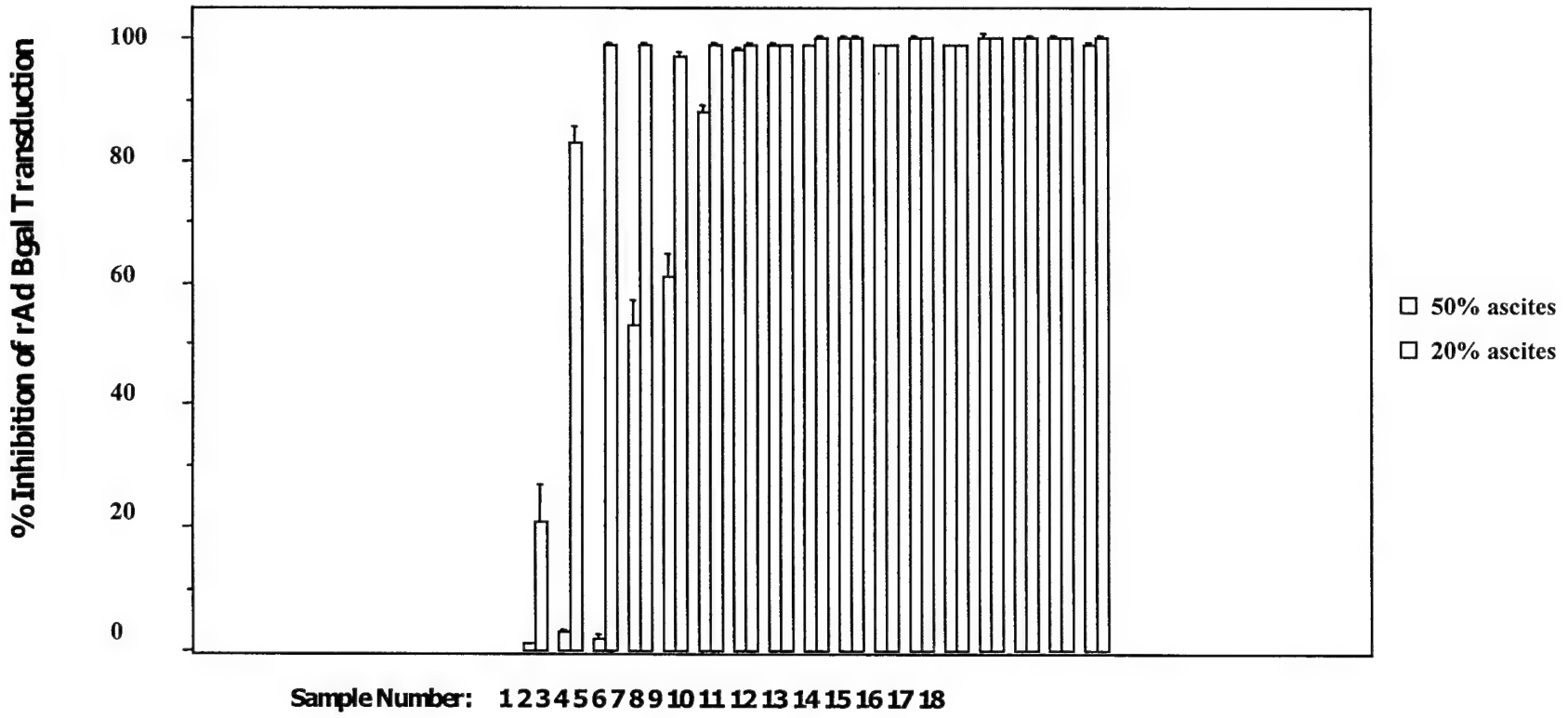


Figure 1B. Inhibition of rAd BGal Transduction by Malignant Effusions

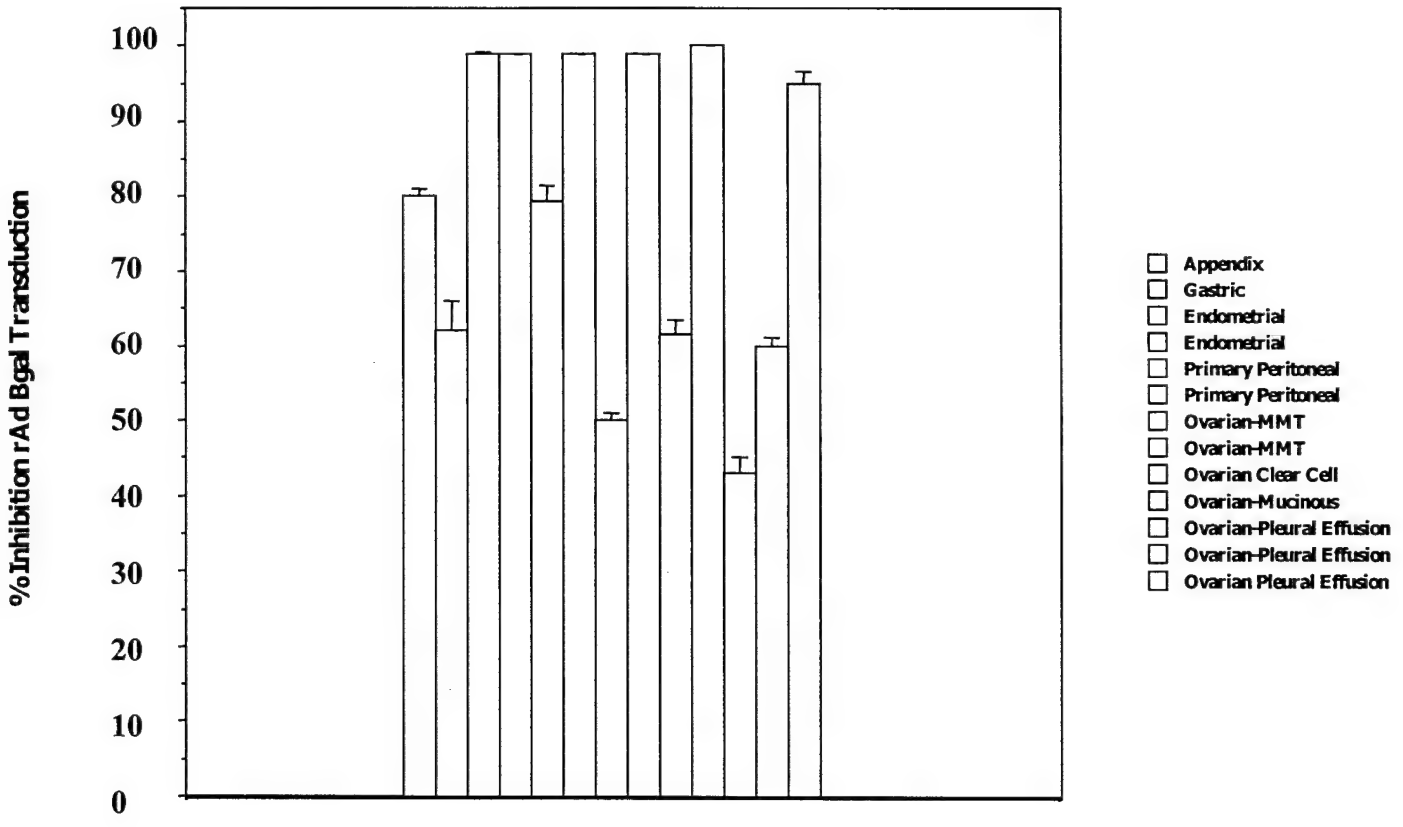


Figure 2A. Dose-dependent Inhibition of Adenovirus Transduction by Ovarian Cancer Ascites Compared to Tumor Cell-Conditioned Media

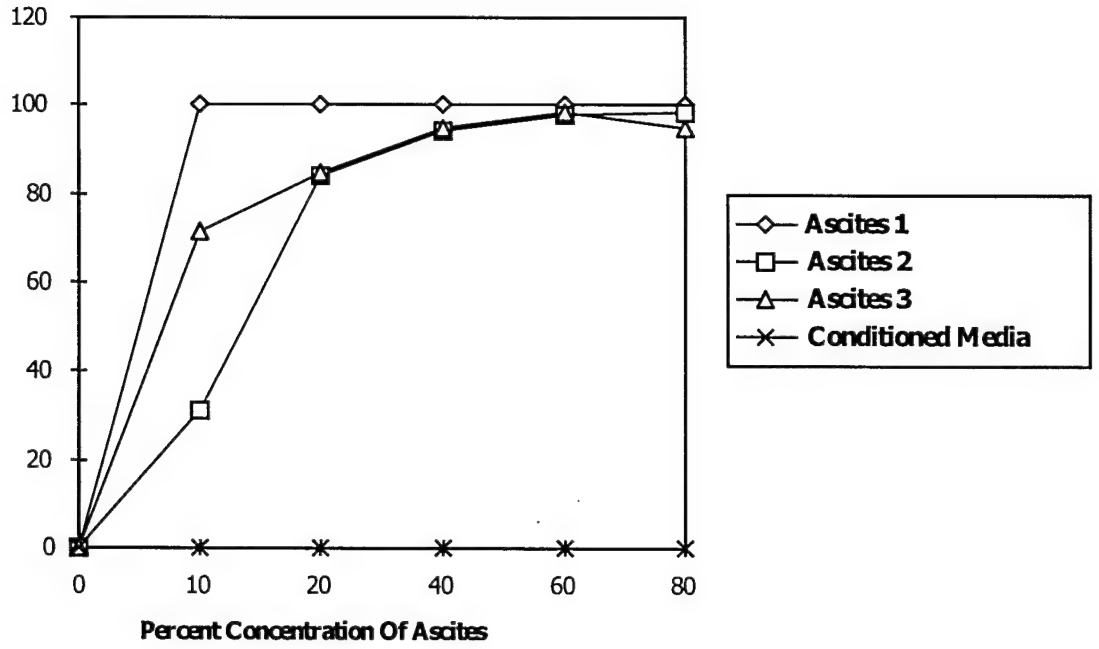


Figure 2B: Effect of Ascites (T808) on rAd Bgal Transduction of MDA-MB-231 Cells

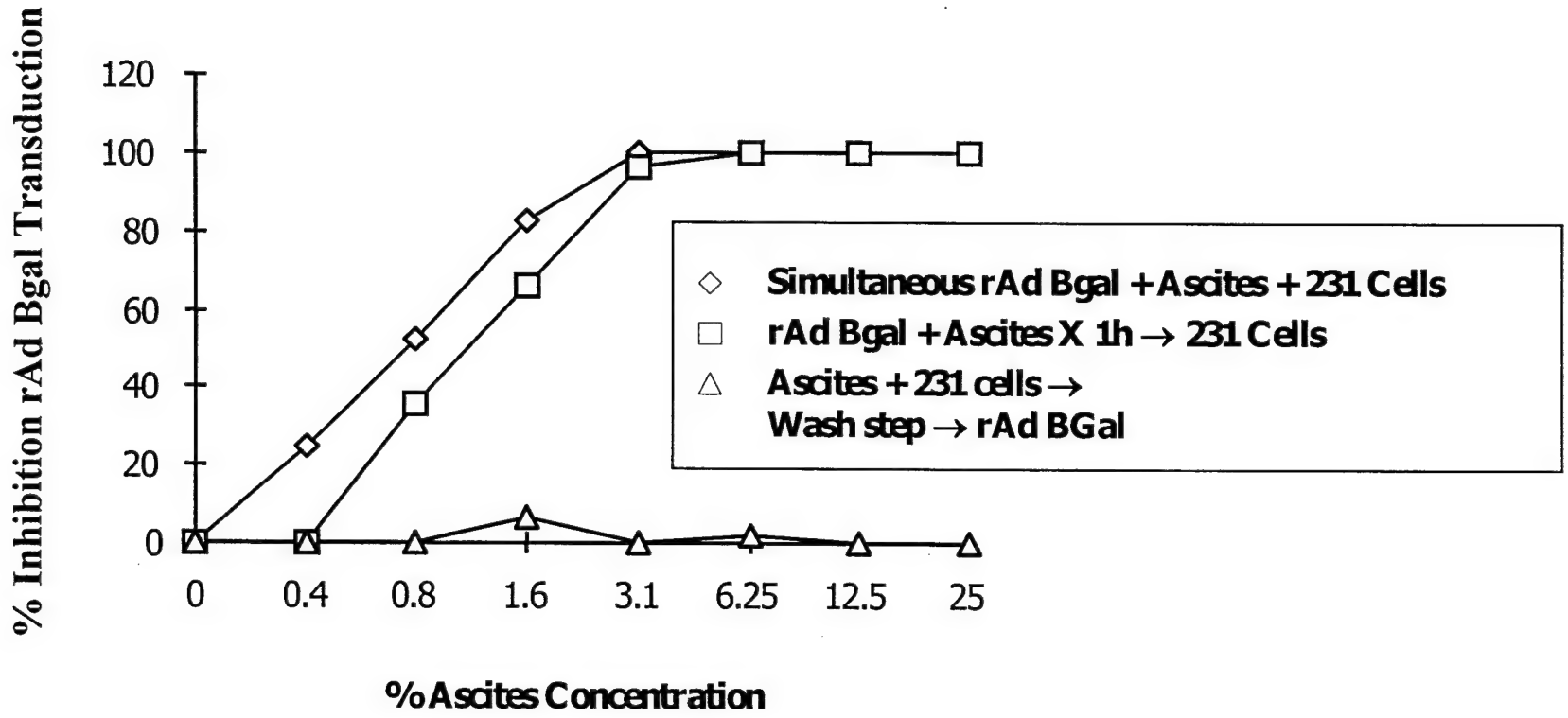


Figure 3. Inhibition of rAd BGal Transduction in Matched Tumor/Ascites Samples

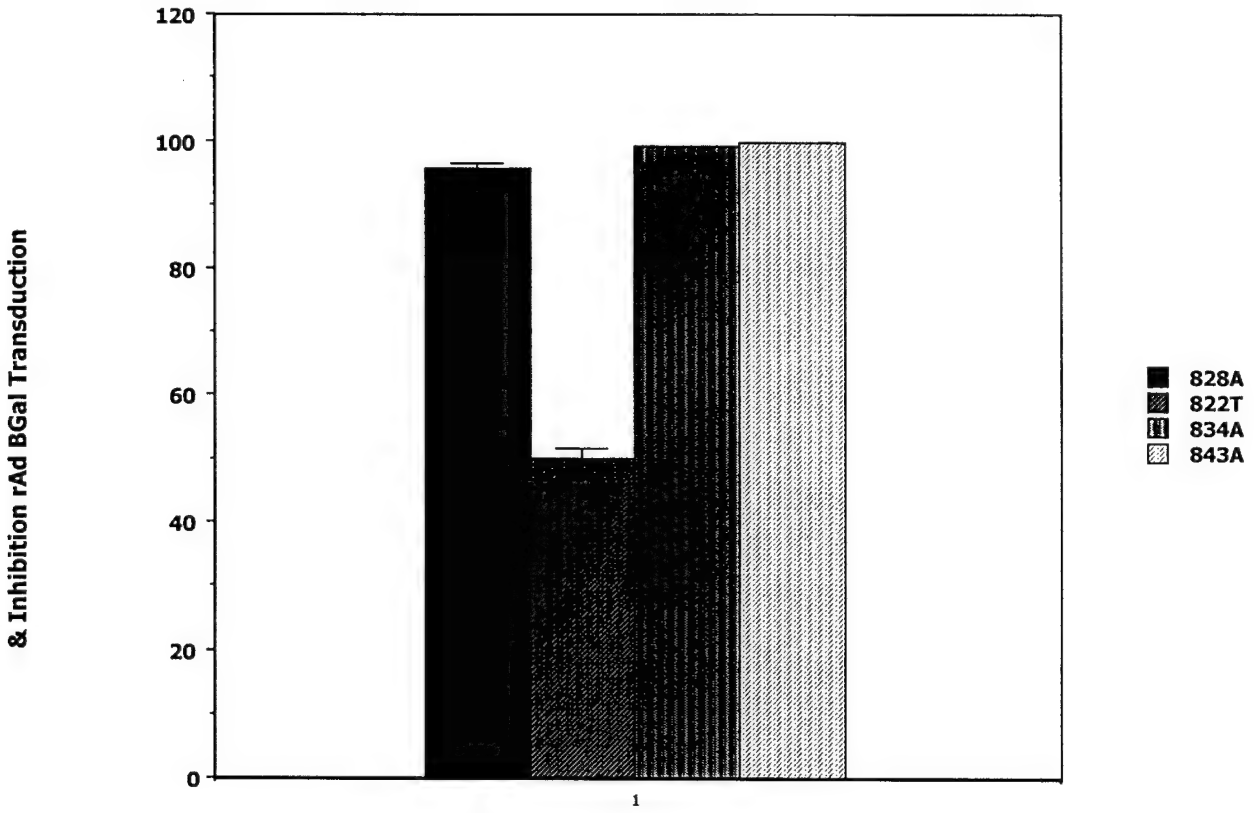


Figure 4. Separation of Anti-Ad Activity by Protein A/G Column Chromatography

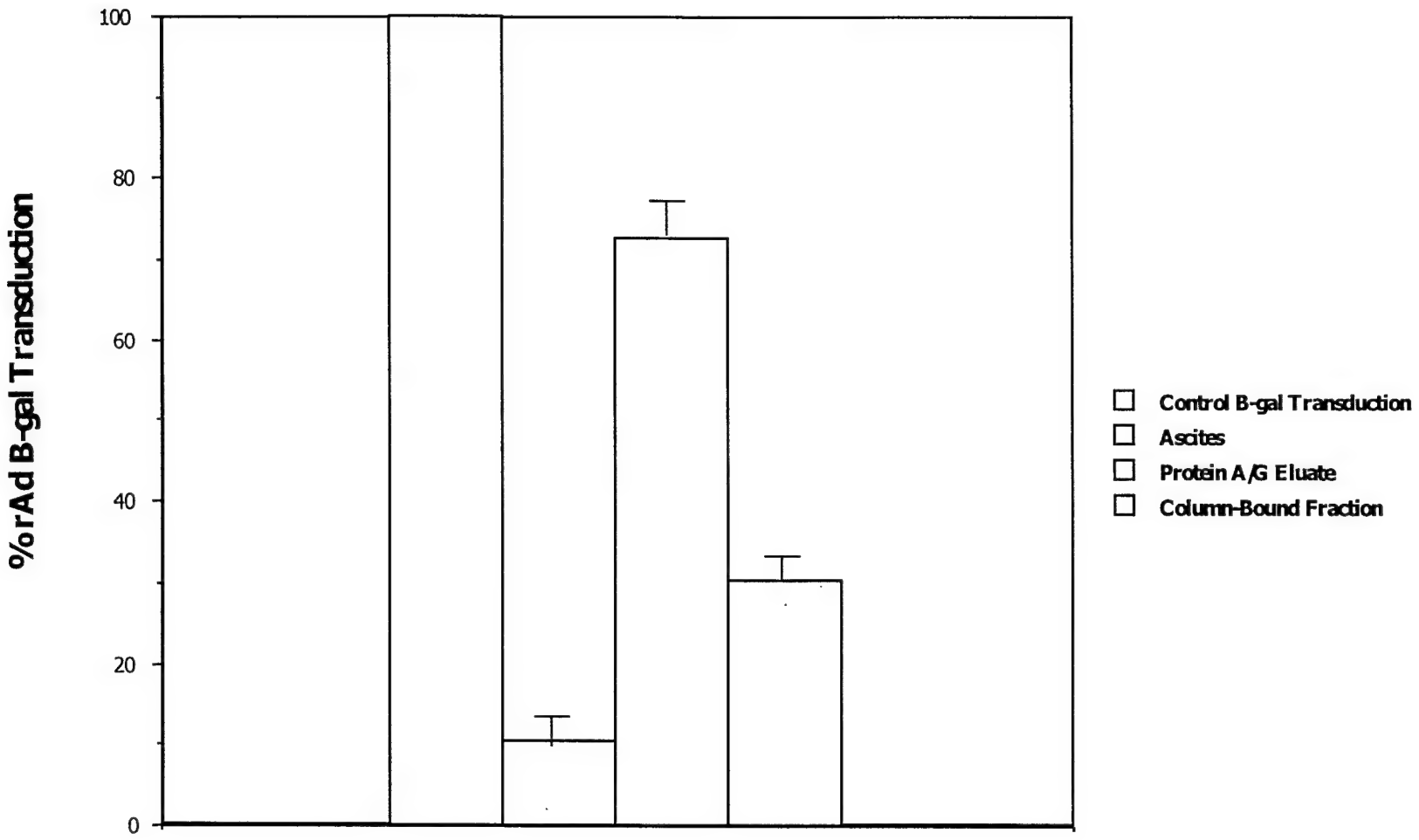


Figure 5A. Chromatography Profile Of Ascites T808 On DEAE-C Column And The Effect On Beta-gal Transduction

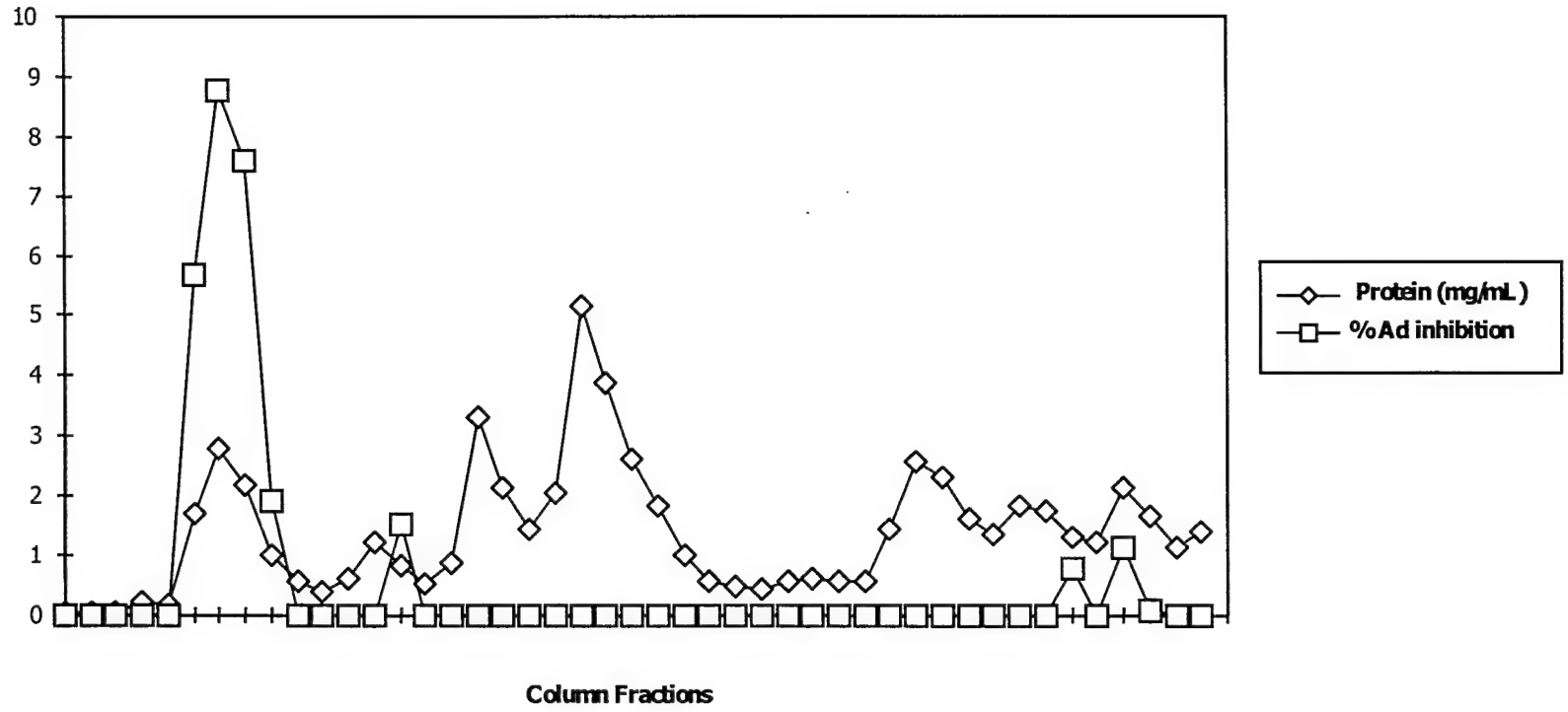
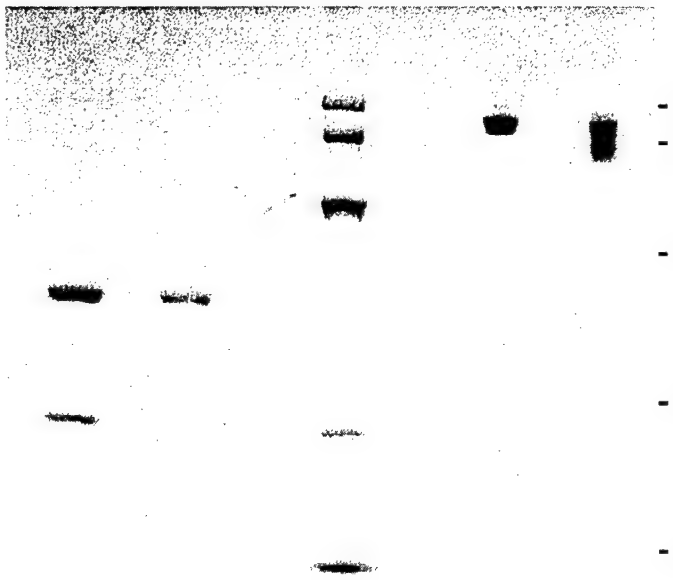
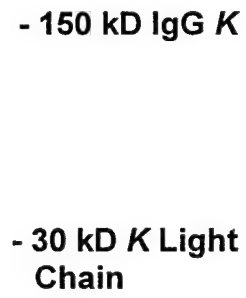


Figure 5B/C. Characterization of Ad Inhibitory Fraction Following Ion Exchange Chromatography of Ascites Yields IgG-*K*

B. Native Gel



C. Anti-IgG Western



Sample Conditions:

Reduced **Non-Reduced** **Reduced** **Non-Reduced**

Table 1. Effect of 20% mouse tumor ascites on adenovirus transduction efficiency following Ad immunization.

Tumor	Mouse	Immunized	% Cells Transduced	Anti-Ad Antibody Titer	Neutralizing Antibody titer
SK-OV-3	SCID	No	99.5 ± 0.4	Negative	Negative
MidT2-1	SCID	No	61.5 ± 3.0	Negative	Negative
MidT2-1	FVB	No	61.9 ± 3.6	Negative	Negative
MidT2-1	FVB	Yes X 1	0.43 ± 0.25	1:2560	1:640
MidT2-1	FVB	Yes X 5	1.5 ± 0.6	1:>2560	1:16000

Association of Urokinase-Type Plasminogen Activator and Its Inhibitor with Disease Progression and Prognosis in Ovarian Cancer[1]

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ABSTRACT

Purpose: **Urokinase-type plasminogen activator (uPA) and its inhibitor, plasminogen activator inhibitor (PAI)-1, have been shown to be related to poor prognosis in a variety of malignant solid tumors. Studies on the prognostic relevance of uPA and PAI-1 in ovarian cancer, however, have been inconclusive. The current study tests the hypothesis that elevated expression of uPA and PAI-1 is associated with prognosis and disease progression.**

Experimental Design: **uPA and PAI-1 were prospectively measured by quantitative ELISA in tumor samples from 103 ovarian cancer patients (82 primary invasive epithelial carcinomas, 9 low malignant potential tumors, and 12 recurrent ovarian carcinomas).**

Results: **uPA but not PAI-1 levels were consistently associated with malignant progression, with levels increased from low malignant potential tumors to primary tumors (uPA, $P = 0.04$; PAI-1, $P = 0.019$), from early to advanced disease stages (uPA, $P = 0.014$; PAI-1, $P = 0.23$), and from primary to intra-abdominal metastatic tumors (uPA, $P = 0.001$; PAI-1, $P = 0.16$). High uPA and PAI-1 levels were associated with residual tumor volumes of >1 cm ($P = 0.001$ and $P = 0.016$, respectively). Among invasive International Federation of Gynecologists and Obstetrician stages I–IV tumors, elevated levels of uPA (>5.5 ng/mg) and PAI-I (>18.8 ng/ml) were associated with a shortened progression-free survival (uPA, $P = 0.003$; PAI-1, $P = 0.039$) and overall survival (uPA, $P = 0.0002$; PAI-1, $P = 0.007$). In multivariate analysis, uPA retained prognostic independence for progression-free survival ($P = 0.037$) and overall survival ($P = 0.006$).**

Conclusions: **These data suggest that the uPA/PAI-1 axis may play an important role in the intra-abdominal spread and reimplantation of ovarian cancer cells. The prognostic relevance of uPA and PAI-1 supports their possible role in the malignant progression of ovarian cancer.**

INTRODUCTION

Ovarian cancer is the leading cause of death from gynecological malignancies and the fourth leading cause of cancer deaths among American women. Little is known about the molecular biology underlying the metastatic process of intra-abdominal dissemination in ovarian cancer. Invasion and metastasis of solid tumors requires proteolytic enzymes that degrade the extracellular matrix and basement membranes (1). Among the proteases involved are the plasminogen activators, of which uPA[4] and/or its inhibitor, PAI-1, have been suggested to play a central role (2–4). Binding of uPA to the uPA receptor (CD87) activates the protease and catalyzes the conversion of plasminogen to plasmin, which subsequently activates type IV collagenase (3), or directly degrades extracellular matrix proteins such as fibrin, lamin, laminins, and proteoglycans (4). The enzymatic activity of uPA is regulated by the plasminogen activator inhibitors, PAI-1 and PAI-2 (5, 6). Both uPA and PAI-1 have been associated with disease outcome as statistically independent prognostic markers in breast (7–9), lung (10), colon (11), kidney (12), and gastrointestinal (13) cancers.

In ovarian cancer, significantly elevated uPA and PAI-1 levels have been described (14–16), however, studies analyzing the clinical impact of uPA and PAI-1 in ovarian cancer have reported inconclusive results, with studies either claiming prognostic importance of PAI-1 (17, 18) or uPA (19), or demonstrating no prognostic relevance for either uPA or PAI-1 (16). The studies with significant results were either performed with patient subsets of advanced disease stages only (17, 18), or without multivariate analysis (19). On the basis of these limitations, the current study was designed with the objective of analyzing the prognostic relevance of uPA and PAI-1 on PFS or OS in uni- and multivariate analyses among patients with all

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[4] The abbreviations used are: uPA, urokinase-type plasminogen activator; PAI, plasminogen activator inhibitor; FIGO, International Federation of Gynecology and Obstetrics; PFS, progression-free survival; OS, overall survival; LMP, low malignant potential.

Table 1 Patient and disease characteristics in invasive primary epithelial ovarian cancers (n = 82)

	N	Percentage
Age		
Median	56	
Range	34–82	
Stage		
FIGO I	12	14.6
FIGO II	6	7.3
FIGO III	50	61.0
FIGO IV	14	17.1
Histology		
Serous	53	64.6
Mucinous	10	12.2
Endometroid	15	18.3
Undifferentiated	3	3.7
Clear cell	1	1.2
Grading		
G1	8	9.8
G2	28	34.1
G3	42	51.2
G4	4	4.9
Hormone receptor status (n = 70)		
ER-negative	27	38.6
ER-positive	43	61.4
PR-negative	41	57.1
PR-positive	29	42.9
uPA		
≤5.5	64	78.0
>5.5	18	22.0
PAI-1		
≤18.8	41	50
>18.8	41	50

disease stages as a cohort representative of primary ovarian cancer in general.

PATIENTS AND METHODS

Patients. One hundred and three consecutive patients (1993–1997) who were treated for ovarian carcinoma at the Department of Obstetrics and Gynecology of the University of Munich, Klinikum Grosshadern, Munich, Germany were enrolled in this study. Complete surgical staging was followed by standard operative procedures, including a bilateral salpingo-oophorectomy, total abdominal hysterectomy, retroperitoneal pelvic and periaortic lymphadenectomy, and partial resection of the small or large intestine, diaphragmatic peritoneum, or upper abdominal surgery if indicated in advanced disease. Ovarian cancer disease was classified according to the FIGO staging system. Postoperative macroscopically visible tumor was the criterion for defining the presence or absence of residual tumor. The tumors studied included 82 primary invasive epithelial carcinomas, 9 LMP tumors, and 12 recurrent ovarian carcinomas. The patient and disease characteristics of the 82 primary invasive carcinomas are summarized in Table 1. Complete follow-up information was available for 80 of these patients.

Sixty-nine of 82 patients with primary ovarian cancer received platinum-based chemotherapy. Of the remaining 13 patients who received a single-drug therapy (n = 2) or no adjuvant treatment (n = 11), most had early stage grade 1 carcinomas (n = 8) or unfavorable health conditions (n = 5). During initiation of the study, standard chemotherapy for primary ovarian cancer was carboplatin/cyclophosphamide with subsequent paclitaxel-containing regimens. A maximum of six cycles of chemotherapy was administered. Computed tomography scans of the abdomen were performed when disease progression was suspected on the basis of gynecological examination, vaginal ultrasound, patient symptoms, or increases in serum tumor markers. Disease progression was defined as a radiologically (computed tomography scan or nuclear magnetic resonance) proven disease recurrence or progression. Second-look procedures were not performed in this cohort. CA 125 and CA 72-4 levels were measured every three months by enzyme immunoassay (Abbott Laboratories, Chicago, IL) and RIA (Centocore, New York, NY), respectively. Median follow-up was 17 months (range, 1–55 months) for all patients. This study was performed after approval by the local Human Investigations Committee of the University of Munich, Munich, Germany. Informed consent was obtained from each patient or patient guardian.

Methods. Biopsies were obtained during surgery, and tissue sections were analyzed by histological assessment in all cases. The remainder of each sample was stored at −198°C in liquid nitrogen until use. Subsequently, frozen specimens of 500 mg wet weight were pulverized with a microdismembrator (Braun-Melsungen, Melsungen, Germany), suspended in 2 ml of Tris-buffered saline containing 1% Triton X-100 detergent (Sigma Chemical Co., Munich, Germany), and incubated at 4°C for 12 h, with subsequent ultracentrifugation at 100,000 × g for 45 min. Quantitative levels of uPA and PAI-1 were prospectively measured in the supernants using ELISA kits, as described (Ref. 20; Imubind uPA and Imubind PAI-1; American Diagnostica, Greenwich, CT). Briefly, the uPA ELISA uses a murine monoclonal capture antibody directed to the uPA β-chain, thus detecting all forms of human uPA and uPA complexes with PAI-1. The detection system uses a biotinylated antibody of uPA α-chain specificity. The PAI-1 ELISA uses a murine monoclonal capture antibody directed to active and inactive PAI-1 and PAI-1 complexes. The detection system uses a biotinylated antibody directed to an epitope that is noncompetitive with the above capture/binding site. Antigen concentrations for uPA and PAI-1 were measured in terms of ng/mg protein. Protein concentrations were measured using the protein assay reagent method (Pierce, Rockford, IL). Assays for estrogen and progesterone receptor content were performed with enzyme immunoassays (ER-EIA and PR-EIA, Abbott Laboratories, Chicago, IL), as described (21).

Statistical Analysis. Statistical analysis was performed using the SPSS statistical software program. Univariate and multivariate analyses were performed by the log-rank test and Cox's regression analysis, respectively. Two group comparisons assuming equal variance were performed using Student's t test (two-tailed). Nonparametric methods were used (Mann-Whitney U test) for non-normally distributed data. Ps of <0.05 were considered to be significant. The cutoff values of uPA and PAI-1 were calculated by log-rank statistic. A cutoff value for uPA and PAI-1 was identified that provided the maximum separation of patients with distinct prognosis with regards to PFS and OS. Survival curves were analyzed by the Kaplan-Meier method (22).

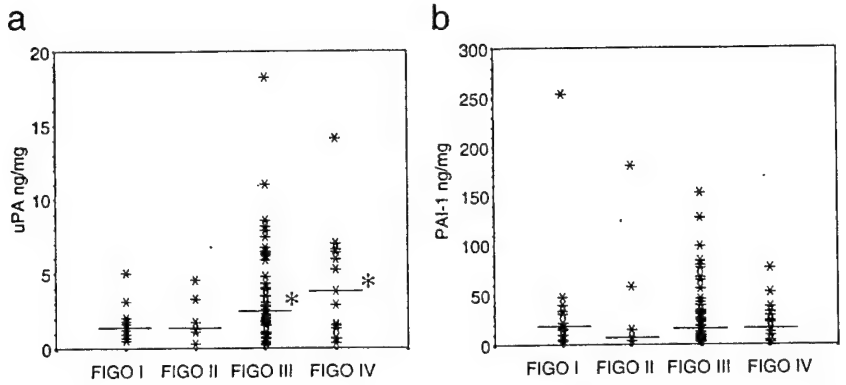

Fig. 1 Comparison of uPA (*A*) and PAI-1 (*B*) levels for all patients with primary ovarian cancer according to FIGO stage. Patients with advanced disease stages had significantly higher uPA but not PAI-1 concentrations compared with patients with early disease stages (uPA ng/mg protein, mean values ± SD; median): FIGO I, 1.7 ± 1.3 (1.5); FIGO II, 2.0 ± 1.6 (1.5); FIGO III, 3.7 ± 3.3 (2.5); FIGO IV, 4.2 ± 3.8 (3.4); $P = 0.014$; (PAI-1, ng/mg protein, mean values ± SD; median): FIGO I, 40.7 ± 68.7 (19.4); FIGO II, 44.7 ± 69.9 (10.7); FIGO III, 30.1 ± 33.3 (19.0); FIGO IV, 23.6 ± 21.5 (19.5); $P = 0.23$. *, $P = 0.014$ for FIGO stages III and IV compared with FIGO stages I and II. *Lines*, the median values.

RESULTS

uPA and PAI-1 concentrations were prospectively measured in tumor samples from 82 patients with primary ovarian cancer, 9 patients with LMP tumors, and 12 patients with recurrent ovarian carcinomas. Patient and disease characteristics of primary ovarian cancer patients are shown in Table 1. The expression of uPA in primary cancers was significantly associated with higher uPA concentrations among patients with higher FIGO stage disease [uPA ng/mg protein, mean values ± SD (median): FIGO I, 1.7 ± 1.3 (1.5); FIGO II, 2.0 ± 1.6 (1.5); FIGO III, 3.7 ± 3.3 (2.5); FIGO IV, 4.2 ± 3.8 (3.4); $P = 0.014$; Fig. 1*A*]. However, no significant association between PAI-1 concentrations and FIGO stage was found [PAI-1 ng/mg protein, mean values ± SD (median): FIGO I, 40.7 ± 68.7 (19.4); FIGO II, 44.7 ± 69.9 (10.7); FIGO III, 30.1 ± 33.3 (19.0); FIGO IV, 23.6 ± 21.5 (19.5); $P = 0.23$; Fig. 1*B*]. uPA and PAI-1 concentrations were significantly higher among primary ovarian cancers (FIGO I; $n = 12$) compared with LMP tumors [$n = 9$; uPA (mean ± SD)], 1.7 ± 1.2 *versus* 1.0 ± 1.2 ng/mg protein; $P = 0.04$; PAI-1 (mean ± SD), 40.7 ± 68.7 *versus* 9.0 ± 3.0 ng/mg protein; $P = 0.019$]. uPA concentrations among primary ovarian cancer patients (FIGO I-IV) were comparable with those measured in recurrent ovarian cancers [uPA (mean ± SD), 3.4 ± 3.1 *versus* 4.0 ± 2.9 ng/ml protein ($P = 0.5$)]; however, PAI-1 concentrations were significantly lower among recurrent ovarian cancers compared with primary ovarian cancers [PAI-1 (mean ± SD), 17.7 ± 15.2 *versus* 31.6 ± 41.5 ng/mg protein ($P = 0.035$)]. Among patients with advanced disease stages (FIGO III and IV), uPA and PAI-1 concentrations were compared between primary tumor sites ($n = 42$) and sites of metastases ($n = 21$). Intra-abdominal metastasis demonstrated significantly higher uPA but not PAI-1 concentrations [uPA (mean ± SD), 5.5 ± 3.8 *versus* 2.9 ± 2.8 ng/mg protein; $P = 0.001$; PAI-1 (mean ± SD), 33.8 ± 32.3 *versus* 24.8 ± 29.8 ng/mg protein; $P = 0.16$]. The relationship of uPA to PAI-1 among all ovarian cancer patients ($n = 103$) was analyzed and is shown in Table 2 and Fig. 2. The data demonstrate a significant positive association between uPA and PAI-1 as dichotomized variables or as continuous variables (χ^2 test, $P = 0.015$; Pearson correlation, $r = 0.22$; $P = 0.02$; Spearman's correlation, $r = 0.41$; $P < 0.001$). Ovarian cancer patients (FIGO I–IV) with elevated uPA levels (>5.5 ng/mg) also demonstrated significantly higher mean PAI-1 concentrations compared with those with lower uPA levels (PAI-1, 44 ± 32 *versus* 28 ± 43 ng/mg protein; $P = 0.003$).

Table 2 Significant association between uPA and PAI-1 for primary ovarian cancer patients ($n = 103$) using the defined cut-off value for uPA (5.5 ng/mg) and PAI-1 (18.8 ng/mg)[a]

	uPA-positive	uPA-negative	N
PAI-1-positive	15 (75%)	32 (39%)	47
PAI-1-negative	5 (25%)	51 (61%)	56
Total	20 (100%)	83 (100%)	100

[a] chi square; $P = 0.003$.

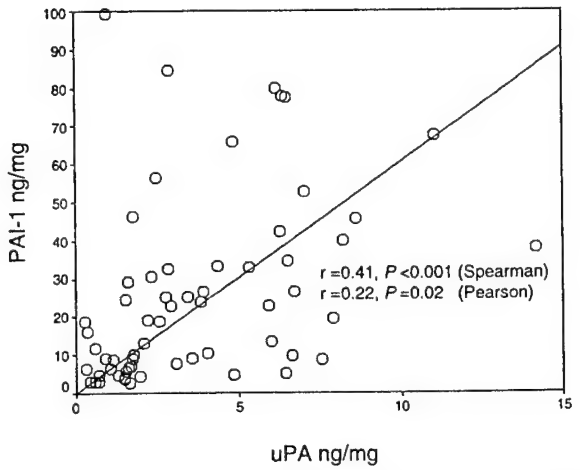

Fig. 2 Association between uPA and PAI-1 concentrations as continuous variables among 103 ovarian cancer patients [$r = 0.22$; $P = 0.02$ (Pearson); $r = 0.41$; $P < 0.001$ (Spearman)].

Table 3 Uni- and multivariate analyses of prognostic factors for PFS and OS in 80 patients with ovarian cancer, FIGO stages I–IV
The following parameters were included in the analyses: tumor stage (FIGO Stages I and II *versus* Stages III and IV), residual tumor (presence or absence), age (≤56 years *versus* >56 years), uPA (≤5.5 *versus* >5.5 ng/mg protein), PAI-1 (≤18.8 *versus* >18.8 ng/mg protein), and grading (G1, G2, G3, or G4).

	*P*s for PFS		*P*s for OS	
	Univariate	Multivariate	Univariate	Multivariate
FIGO stage	0.005	0.95	0.016	0.88
Residual tumor volume	<0.0001	0.0005	0.0003	0.025
uPA	0.003	0.037	0.0002	0.006
PAI-1	0.039	0.31	0.007	0.582
Age	0.059	0.0008	0.15	0.018
Grading	0.0002	0.962	0.004	0.882

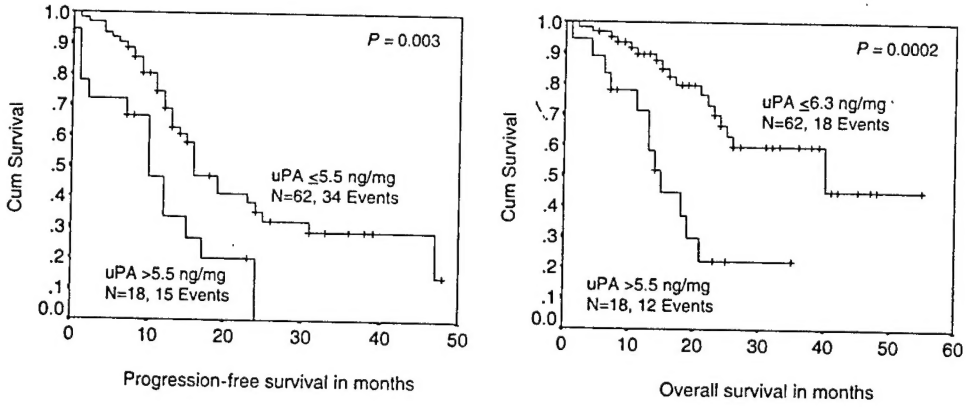

Fig. 3 Kaplan-Meier plots for PFS and OS of ovarian cancer patients, FIGO stages I–IV. Patients with uPA levels below the cutoff value (5.5 ng/mg protein) had improved median PFS and OS compared with patients with elevated levels (PFS, 16 *versus* 10 months; $P = 0.003$; OS, 40 *versus* 15 months; $P = 0.0002$).

Evaluation of uPA and PAI-1 on Prognosis (PFS and OS). Among invasive cancers of all stages, residual tumor volume, FIGO stage, and grading were significant prognostic factors for both PFS and OS in univariate analyses (Table 3). Patients with early disease stages (FIGO I and II; $n = 18$) had a significantly better prognosis than patients with advanced disease stages (FIGO III and IV; $n = 62$; median PFS, 47 *versus* 13 months; $P = 0.005$; median OS not reached for FIGO stages I and II patients *versus* 24 months for FIGO stages III and IV patients; $P = 0.016$). Likewise, patients with no residual tumor volume ($n = 28$) had a marked advantage in prognosis compared with patients with residual volume ($n = 52$; median PFS, 47 *versus* 12 months; $P < 0.0001$; median OS not reached for patients without residual disease *versus* 21 months for patients with residual disease; $P = 0.0003$). Age (≤56 or >56 years) demonstrated borderline significance for PFS ($P = 0.059$) and was not significant for OS ($P = 0.15$).

To evaluate the prognostic impact of uPA and PAI-1 levels on prognosis, we identified optimized cutoff values for separation of patients with distinct prognosis, using univariate analysis. A uPA value of 5.5 ng/mg provided the maximum separation of patients with regards to PFS and OS (log-rank test; $P = 0.003$ and 0.0002, respectively). A PAI-1 value of 18.8 ng/mg similarly provided the maximum separation of patients with regards to PFS and OS (log-rank test; $P = 0.039$ and $P = 0.007$, respectively). Among invasive cancers of all stages, patients with uPA concentrations below 5.5 ng/mg protein ($n = 62$) had an improved median PFS and OS compared with patients with elevated levels ($n = 18$; median PFS, 16 *versus* 10 months, $P = 0.003$; median OS, 40 *versus* 15 months, $P = 0.0002$; Fig. 3). Similarly, patients with PAI-1 levels below 18.8 ng/mg protein ($n = 40$) had an improved PFS and OS compared with patients with elevated levels ($n = 40$; median PFS, 19 *versus* 13 months, $P = 0.039$; median OS not reached for PAI-1-negative patients *versus* 21 months for PAI-1-positive patients, $P = 0.007$; Fig. 4). uPA concentration retained prognostic significance for OS in patients with residual tumor ($n = 54$; median OS, 24 *versus* 14 months; $P = 0.012$) and achieved borderline significance among patients with no residual tumor ($n = 28$; mean OS, 53 *versus* 20 months; $P = 0.06$). Neither estrogen and progesterone receptor status nor the volume of ascites (>500 *versus* ≤500 ml) or CA 125 values (>35 *versus* ≤35 units/ml) were significant prognostic factors in this cohort (data not shown). In multivariate analysis of patients with FIGO stages I–IV disease, which included the parameters of the FIGO stage, residual tumor volume, uPA, PAI-1, age and grading, the parameters of residual tumor volume, uPA, and age remained independent prognostic factors for PFS and OS (Table 3).

To compare this study with previous studies in which only patients with advanced disease stages were analyzed, the prognostic significance of uPA and PAI-1 in the subset of FIGO stages III and IV patients ($n = 62$) was analyzed. Among these invasive cancers, residual tumor volume ($P = 0.0007$), age ($P = 0.005$), and uPA ($P = 0.037$) were significant prognostic factors for PFS, whereas FIGO stage ($P = 0.10$) was only of borderline significance. FIGO stage ($P = 0.024$), residual tumor volume

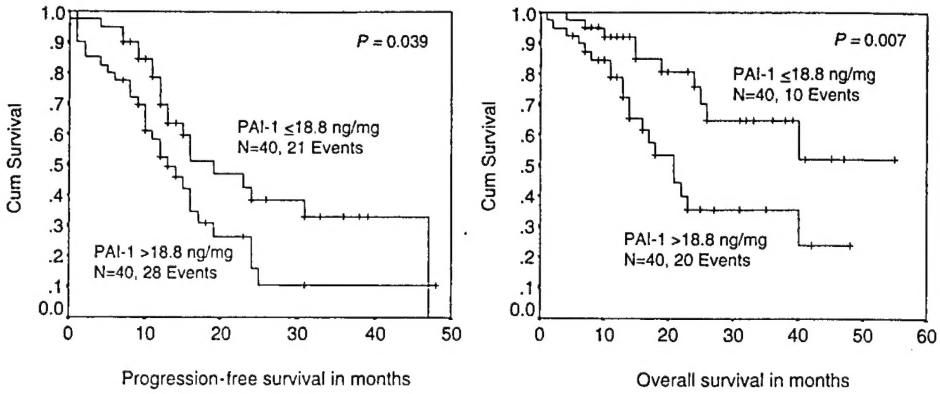

Fig. 4 Kaplan-Meier plots for PFS and OS of ovarian cancer patients, FIGO stages I–IV. Patients with PAI-1 levels below the cutoff value (18.8 ng/mg protein) had improved median PFS and OS compared with patients with elevated levels (PFS, 19 *versus* 13 months; $P = 0.039$; OS, median OS not reached for patients with PAI-1 levels ≤18.8 n/mg protein *versus* 21 months; $P = 0.007$).

($P = 0.012$), and uPA ($P = 0.003$) were significant prognostic factors for OS, whereas age ($P = 0.056$) and PAI-1 ($P = 0.066$) were of borderline significance. Interestingly, FIGO stage III or IV patients with optimal surgical cytoreduction ($n = 28$) had significantly lower uPA and PAI-1 concentrations than those with higher volumes (>1 cm; $n = 36$) [uPA (mean ± SD), 2.4 ± 2.4 *versus* 4.9 ± 3.7 ng/mg protein, $P = 0.001$; PAI-1 (mean ± SD), 22.9 ± 32.1 *versus* 33.1 ± 30.0 ng/mg protein, $P = 0.016$]. This suggests that the inability to optimally debulk patients could be related to the increased proteolytic activity in tumors observed in patients with higher residual volumes. In multivariate analysis of advanced ovarian cancer, including FIGO stage, residual tumor volume, age, and uPA, the parameters of residual tumor volume (PFS, $P = 0.0004$; OS, $P = 0.025$), uPA (PFS, $P = 0.07$; OS, $P = 0.007$) and age (PFS, $P = 0.0006$; OS, $P = 0.025$) retained independent prognostic importance.

DISCUSSION

In the present study, uPA concentrations were significantly higher in invasive tumors compared with LMP tumors, which have been recognized as a separate entity, as the clinical course of these tumors is far more favorable when compared with their invasive counterparts (23). Similarly, uPA levels were higher in metastatic lesions as compared with their respective primary tumors. Increasing levels of uPA were also significantly associated with advanced disease stages and with the amount (>1 cm) of residual tumor. Taken together, these findings demonstrate that uPA is associated with the malignant progression of epithelial ovarian cancer. The results are consistent with the hypothesis that elevated levels of uPA may contribute to invasiveness and metastasis of ovarian cancer. In contrast, PAI-1 content was not correlated with disease stage, which is in accordance with previous reports (16, 17), and no significant difference was found in PAI-1 content between primary and metastatic ovarian cancers as compared with uPA.

The present study is the first study to evaluate the prognostic significance of uPA and PAI-1 in uni- and multivariate analyses in a representative cohort of primary ovarian cancers of all stages. The level of uPA was an independent prognostic marker for both PFS and OS in multivariate [illegible] the cutoff values established for this cohort. Consistent with previous reports, we also confirmed the independent prognostic relevance of residual tumor volume and age in ovarian cancer (24–28). This is the first study to demonstrate the independent prognostic relevance of uPA in a nonselected group of ovarian cancer patients. In a recent report on 77 patients with primary ovarian cancer (FIGO stages I–III), Hoffmann *et al.* (19) were also able to demonstrate prognostic relevance of uPA for OS; however, they only performed univariate analysis. In contrast, van der Burg *et al.*, (16) who assessed uPA and PAI-1 among 90 patients ranging from stage I to stage IV disease, reported no correlation of uPA and PAI-1 with PFS or OS. The negative findings of that study possibly are attributable to the different laboratory assays, extraction procedures, and cutoff values used, as van der Burg *et al.* based median values as cutoff values for uPA and PAI-1 and measured uPA and PAI-1 concentrations in cytosols routinely prepared for ER and PR determinations.

Two previous studies support a poor prognosis associated with high PAI-1 content (17, 18). Chambers *et al.* (17) determined PAI-1 levels by immunohistochemistry in samples from 119 patients with FIGO stages I–IV disease, and PAI-1 was an independent prognostic factor among the 99 patients with FIGO stages III or IV disease; however uPA was not included in this analysis. Kuhn *et al.* (18) recently demonstrated the importance of PAI-1 as an independent prognostic marker for survival by assessing PAI-1 by ELISA among 84 ovarian cancer patients with FIGO stage IIIc disease. In the present study, the prognostic relevance of high PAI-1 levels for PFS and OS was confirmed in univariate analysis only when all disease stages were included in the analysis; however, this was not the case among the subset of patients with advanced disease for PFS, and for OS the effect was of borderline significance ($P = 0.066$), possibly because of the small sample size ($n = 64$) in subset analysis.

Not only did the previously mentioned studies use different extraction procedures or assay methods and different antibodies, but, also, different cutoff values were applied to separate patients into low- *versus* high-risk groups. Hoffmann *et al.* (19), who analyzed a comparable cohort (FIGO stages I–III) of primary ovarian cancer patients, detected uPA and PAI-1 levels in tissue pellets and selected a slightly lower

optimized cutoff value of 4.8 ng/ml for uPA. However, they did indicate that the uPA concentrations were ~30% lower if detection was performed in tissue pellets, as done in their study, compared with tissue homogenates, which were used in the present study.

The optimal cutoff value for uPA in this study was 5.5 ng/mg protein, meaning that 18 of 82 patients (22%) whose tumors revealed elevated uPA concentrations had a significantly shorter OS compared with those with lower uPA levels. To further establish the prognostic impact of uPA in ovarian cancer, the previously described cutoff value of 4.8 ng/ml protein for uPA was analyzed (data not shown). This cutoff value also provided clinically significant results in this study population, in which 22 of 82 patients (27%) had a significantly shorter survival compared with those with lower uPA concentrations (P = 0.006). uPA also retained independent prognostic significance with the selected cutoff value of 4.8 ng/mg protein in multivariate analysis, including FIGO stage, residual tumor volume, levels of PAI-1, age, and histological grade (P = 0.0088). These data, however, also demonstrate that suitable cutoff values for both uPA and PAI-1 must be further defined and validated in a prospective setting.

This study presents evidence that elevated levels of the uPA protease and, to a lesser extent, its inhibitor, PAI-1, are associated with the capacity of ovarian cancer cells to invade then metastasize in the peritoneum. Additional research, however, is necessary to understand the role of PAI-1 in this process. It has been suggested that PAI-1 can promote internalization of receptor-bound uPA, which allows recycling of the uPA receptor to the cell surface (29). In this way, the proteolytically active areas of the cell surface can be modified by PAI-1, thus regulating directed proteolytic activity of tumor cells (4). It recently has been described that competitive displacement of uPA from the cellular binding sites results in decreased proteolysis *in vitro*. Metastatic capacity was similarly inhibited when animals were given intermittent i.p. injections of uPA/IgG fusion protein capable of displacing uPA activity from the tumor cell surface (30). Assessment of uPA and PAI-1 levels may therefore allow identification of ovarian cancer patients at high risk and provide a rationale for a biologically directed therapy.

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